

Fumonisin exposure biomarkers in humans consuming maize staple diets

by

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Abstract

Fumonisin is a carcinogenic mycotoxin which occurs world-wide in maize and maize-based products intended for human consumption. Consumption of fumonisin-contaminated maize as a staple diet has been associated with oesophageal and liver cancer incidence as well as neural tube defects. This study has confirmed the State of Santa Catarina, Brazil as another region where the consumption of maize contaminated with fumonisins and high oesophageal cancer incidence co-occurs. Since fumonisins exert their main biochemical effect by disruption of the sphingolipid biosynthetic pathway and are implicated in cancer, the role of fumonisin B₁ (FB₁) in FB₁-induced rat hepatocyte nodules was investigated. The current study showed that FB₁ exposure activated sphingosine accumulation in the nodules which could induce the bio-active sphingosine 1-phosphate to provide a selective growth stimulus on subsequent FB₁ exposure. Since the FB₁-induced hepatocyte nodules were not resistant to the disruption of sphingolipid biosynthesis, it was not the mechanism whereby the altered hepatocytes escaped the mitoinhibition of FB₁ and selectively proliferated into hepatocyte nodules. A study in maize subsistence farming communities investigated the sphingosine and sphinganine levels in blood and urine of participants. Fumonisin exposure was assessed in these communities based on fumonisin levels in maize that was concurrently collected from the areas where the participants resided. Subsequently fumonisin exposure was assessed in individuals based on the fumonisin levels in maize collected from each household and by acquiring weighed food records for each member of the household. It was confirmed in both these studies that communities are chronically exposed to fumonisin levels well above the provisional maximum tolerable daily intake determined by the Joint

FAO/WHO Expert Committee on Food Additives. Since the sphinganine and sphingosine levels in blood and urine of the participants exposed to various levels of fumonisin were not significantly different, the sphingoid bases and their ratios could not be established as a biomarker of fumonisin exposure. Therefore, an alternative biomarker of exposure was investigated during studies into a practical cost effective method to reduce fumonisin. The customary maize food preparation practices were assessed in a maize subsistence farming community and subsequently optimised to reduce the fumonisin levels in the maize under laboratory-controlled conditions. Implementation of this optimised and culturally acceptable intervention method of sorting and washing maize in a rural community reduced fumonisin contamination in home-grown maize by 84%. The intervention study attained a 62% reduction in fumonisin exposure based on fumonisin levels in maize-based food and consumption as assessed by 24-h dietary recall questionnaires. The alternative biomarker of fumonisin exposure, urinary FB₁, was investigated during the intervention study. The FB₁ urinary biomarker measured fumonisin intake at the individual level and confirmed the reduction achieved as assessed by food analysis and food intake data. The biomarker was thus well correlated with fumonisin exposure and confirmed the efficacy of the simple and culturally acceptable intervention method. Utilisation of the urinary FB₁ biomarker and the customised hand-sorting and washing of maize to reduce fumonisin exposures has the potential to improve food safety and health in subsistence maize farming communities.

Opsomming

Fumonisien is kankerverwekkende mikotoksiene wat wêreldwyd voorkom op mielies en mielie-verwante produkte bestem vir menslike verbruik. Daar is 'n verband tussen die voorkoms van slukderm en lewer kanker, sowel as neuraalbuisdefekte, in gemeenskappe waar fumonisien-gekontamineerde mielies die stapel voedsel is. Die Brasiliaanse Staat, Santa Catarina is uitgewys as nog 'n area waar hoë voorkoms van slukdermkanker en hoë fumonisin vlakke in mielies gesamentlik voorkom. Aangesien fumonisien verbind word met van kanker en die hoof biochemiese effek die ontwrigting van die sfingolipiedbiosintese weg is, is die rol van fumonisien B₁ (FB₁) in FB₁-geïnduseerde rot hepatosietnodules ondersoek. Die studie het getoon dat FB₁ blootstelling aktiveer sfingosien ophoping in die hepatosietnodules wat moontlik die bio-aktiewe sfingosien 1-fosfaat aktiveer om op daaropvolgende FB₁ blootstellings geselekteerde groei stimulasie te ondergaan. Die FB₁-geïnduseerde hepatosietnodules was nie bestand teen die inhibisie van die sfingolipied biosintese nie en dus nie die meganisme waardeur die veranderde hepatosiete mito- inhibisie van FB₁ vryspring, en selektief ontwikkel in hepatosietnodules nie. 'n Studie in bestaansboerdery gemeenskappe het die sfingosien en sfinganien vlakke in bloed en uriene vergelyk met individuele fumonisien blootstelling. Laasgenoemde is gebaseer op fumonisien vlakke in gekolleekteerde mielies vanuit die deelnemers se huise en aannames vanuit die literatuur. Die opvolg studie in die areas het individuele fumonisien blootstelling bepaal gebaseer op fumonisien vlakke in die mielies van elke huishouding en die inname van mielies deur die voedsel van elke individu te weeg. Albei hierdie studies het bevestig dat die gemeenskappe blootgestel is aan kroniese fumonisien vlakke wat die maksimum toelaatbare

daaglikse inname wat deur die gesamentlike FAO/WHO deskundige komitee op voedsel toevoegsels vasgestel is, oorsake. Aangesien die sfingosien en sfingonien vlakke nie beduidend verskil in bloed of urine van mense wat aan verskillende fumonisien-kontaminasie vlakke blootgestel is nie, kan die lipiedbasisse en hul verhouding nie as 'n biologiese merker vir fumonisien blootstelling bevestig word nie. Dus is 'n alternatiewe biologiese merker vir fumonisien blootstelling ondersoek gedurende 'n studie oor praktiese bekostigbare maniere om fumonisin blootstelling te verlaag. Die tradisionele voedsel voorbereidingspraktyke in 'n bestaansboerdery gemeenskap is bestudeer en onder laboratorium-gekontroleerde toestande aangepas om fumonisien vlakke in die mielies optimaal te verlaag. Die kultureel aanvaarbare intervensie metode, sortering en was van die mielies, is in 'n bestaansboerdery gemeenskap toegepas waar 'n 84% verlaging in fumonisien vlakke van die mielies verkry is. Die intervensie metode het 'n 62% verlaging in fumonisien blootstelling te weeggebring deur fumonisien vlakke in die mieliegebasseerde disse te meet en inname daarvan deur die deelnemers met 24-h diëetkundige vraelyste vas te stel. Gedurende die intervensie studie is urineêre FB₁, die alternatiewe biologiese merker van fumonisien blootstelling, ondersoek. Individuele fumonisien blootstelling data, bepaal met die urineêre FB₁ biomerker, het goed ooreengestem met die voedsel analise en voedsel inname data en het dus die doeltreffendheid van die praktiese kultuur aanvaarbare intervensie metode bevestig. Benutting van die FB₁ urineêre biologiese merker en die optimale sortering en was van die mielies om die fumonisien blootstelling te verlaag het die potensiaal om voedselveiligheid en gesondheid in hierdie bestaansboerdery gemeenskappe aansienlik te verbeter.

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Dedication

Met liefde en dankbaarheid aan my pa, Albertus Johannes, en ma, Elsie Jacoba

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Abbreviations

2-AAF	2-acetylaminofluorene
ASIR	age-standardized incidence rate
bw	body weight
CI	confidence interval
CV	coefficient of variation
DEN	diethylnitrosamine
FB ₁	fumonisin B ₁
FB ₂	fumonisin B ₂
FB ₃	fumonisin B ₃
IARC	International Agency for Cancer
JECFA	Joint FAO/WHO Expert Committee
HPLC-MS	high performance liquid chromatography coupled to mass spectrometry
LC-MS/MS	liquid chromatography coupled to tandem mass spectrometry
LOD	limit of detection
NTD	neural tube defect
MS	mass spectrometry detector
NOEL	no observed effect level
PDI	probable daily intake
PH	partial hepatectomy
PMTDI	provisional maximum tolerable daily intake
S1P	sphingosine 1-phosphate
Sa	sphinganine
So	sphingosine
TFB	total fumonisin (FB ₁ + FB ₂ + FB ₃)
TDI	tolerable daily intake
UFB ₁	Urinary FB ₁
UFB ₁ C	Urinary FB ₁ normalized with urinary creatinine

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Introduction

Objectives of study

- The investigation of fumonisin levels in maize from rural communities with a high prevalence of oesophageal cancer.
- The mechanism of fumonisin inhibition of the sphingoid bases in rat liver nodules.
- The effect of fumonisin on sphingoid bases in blood and urine of communities, as well as individuals in communities, consuming subsistence grown maize.
- The evaluation of an optimized culturally acceptable method to reduce fumonisin contamination in subsistence grown maize by means of an intervention study in a rural community.
- Validation of a urinary biomarker for FB₁ exposure.

Fumonisin, carcinogenic mycotoxins, produced predominantly by *Fusarium verticillioides*, occur widely around the world on maize (*Zea mays*) (Marasas 2001). The major naturally occurring fumonisin analogues in maize and maize-based products intended for human consumption are fumonisin B₁ (FB₁), B₂ (FB₂) and B₃ (FB₃) (Shephard et al., 1996). The contamination of maize with fumonisins is of concern as these mycotoxins cause various animal diseases and occur in maize and maize-based products intended for human consumption (Shephard et al., 1996). In addition, high levels of fumonisins have been found in naturally contaminated maize from areas where high incidences of oesophageal cancer occur, e.g., Centane District, Transkei region of South Africa; Cixian County, Hebei Province, China; and Mazandaran Province, Iran (Chu and Li 1994; Rheeder et al, 1992; Shephard et al., 2000; 2002). Based on current data, the International Agency for Research on

Cancer has classified FB₁ to be possibly carcinogenic to humans (group 2B carcinogen) (IARC, 2002).

The situation in southern Brazil is similar to other regions in the world where high oesophageal cancer incidence and high maize consumption co-occur. Brazil is the third largest producer of maize in the world, of which the southern region is the highest producer and consumer of maize-based products. A considerable portion of this maize crop is produced by small farmers and nearly 25% of the harvest is consumed on these farms. Santa Catarina, Paraná and Rio Grande do Sul States, southern Brazil, have the highest incidences of oesophageal cancer in the country. As fumonisin data in the State of Santa Catarina have not been previously reported, fumonisin levels and *Fusarium verticillioides* contamination of maize collected from different regions in this state were investigated in this study.

Fumonisin exerts its main biochemical effect by inhibiting ceramide synthase, a key enzyme in the de novo sphingolipid biosynthetic pathway, preventing the conversion of sphinganine to dihydroceramide and the reacylation of sphingosine to ceramide (Riley et al., 1994; Wang et al., 1991). The disruption of the sphingolipid biosynthetic pathway elevates the levels of the sphingoid bases and their 1-phosphates and decreases ceramide and more complex sphingolipids, such as sphingomyelin and gangliosides, and their intermediates (Riley et al., 2001; Merrill et al., 2001). Sphingolipids are predominantly found in cellular membranes and are critical for the maintenance of the membrane structure, while complex sphingolipids function as precursors for second messengers and are important in sustaining cellular growth and differentiation (Merrill et al., 2001).

FB₁ inhibits cell proliferation in various cell culture systems as well as in rat liver and kidney (Gelderblom et al., 1996; Riley et al., 2001; Yoo et al., 1992). FB₁-induced disruption of sphingolipid biosynthesis can either induce or prevent apoptosis, depending on the cell type and the relative amounts of the bio-active sphingolipid molecules generated (Desai et al., 2002; Tolleson et al., 1996). In rat liver a carcinogen dose above the initiation threshold induces the appearance of altered hepatocytes which are resistant to the inhibition of proliferation (Solt et al., 1980). These resistant hepatocytes escaped the mitoinhibitory effects of FB₁ on normal hepatocyte growth and selectively proliferate into hepatocyte nodules (Gelderblom et al., 1995; 2001). The exact mechanism involved in the selection of initiated cells by FB₁ is unknown.

An investigation to determine whether hepatocyte nodules are resistant to the inhibitory effect of FB₁ on ceramide synthase was conducted in rats. A further aim was to determine if the resistant hepatocyte nodules would proliferate to a greater extent than normal hepatocytes, which could selectively stimulate their outgrowth. Male Fischer 344 rats were subjected to cancer initiation (FB₁ containing diet or diethylnitrosamine by intraperitoneal injection) and promotion (2-acetylaminofluorene with partial hepatectomy) treatments followed by a secondary FB₁ dietary regimen. Sphinganine and sphingosine levels were determined in control, surrounding and nodular liver tissues of the rats.

The inhibition of ceramide synthase by fumonisin causes sphinganine and sphingosine to a lesser extent, to increase (Riley et al., 2001; Merrill et al., 2001). The resultant increase in the sphinganine/sphingosine ratio occurs prior to changes

in other biochemical markers of cellular injury, and has thus been proposed as a biomarker of fumonisin exposure (Riley et al., 1994). Various animal studies have successfully investigated the sphinganine/ sphingosine ratio as a biomarker of fumonisin exposure in serum, urine, liver, kidney and other tissues (Cai et al. 2007; Castegnaro et al., 1996; Riley et al., 1994; Van der Westhuizen et al. 2001; Howard et al., 2001; Wang et al., 1992). Sphinganine and sphingosine, as well as their ratio, have also been investigated in several human studies in blood and urine, but could not be correlated with fumonisin exposure (Castegnaro et al., 1998; Solfrizzo et al. 2004, Van der Westhuizen et al. 1999; Qiu and Liu 2001).

Since further evidence was required to utilise the sphinganine/sphingosine ratio as a biomarker of fumonisin exposure in humans, a cross sectional study was undertaken in a high (Centane district) and low (Bizana district) oesophageal cancer prevalence area in the former Transkei region of the Eastern Cape Province of South Africa. The rural farmers from these areas consume fumonisin-contaminated maize as a staple diet. Blood and urine samples were collected from male and female volunteers residing in the same areas from which the maize samples were collected. The aim was to compare the sphinganine and sphingosine levels, as well as their ratio, in plasma and urine of participants between the two areas with the contamination level of the maize they consumed collected contemporaneously from these areas.

A further study on the possible applicability of the sphinganine/sphingosine ratio was conducted by measuring individual fumonisin exposure. This assessment of the sphinganine/sphingosine ratio as biomarker of fumonisin exposure in humans, required in addition to the sphinganine and sphingosine levels in blood and/or urine,

the fumonisin levels in the maize and porridge consumed as well as the amount consumed on an individual basis to be determined. This study was conducted in the Centane and Bizana districts of the former Transkei over three consecutive years. Blood and urine samples were collected from male and female participants who donated their maize and maize-based food prepared on the day of collection. Correlation between fumonisin exposure and the sphinganine/ sphingosine ratio would confirm the sphinganine/sphingosine ratio as a biomarker of fumonisin exposure.

In developed countries maize forms a minor part of the diet, whereas in rural communities in South Africa, maize consumption can be as high as 460 g per person per day (Marasas, 2001). Furthermore, subsistence farming communities that consume maize as a staple diet can be exposed to total fumonisin levels of up to 13.8 µg/kg body weight/day. This is of concern as the Joint FAO/WHO Expert Committee on Food Additives (JECFA) has determined a group provisional maximum tolerable daily intake (PMTDI) for fumonisin B₁, B₂ and B₃, alone or in combination, of 2 µg/kg body weight/day (Bolger et al., 2001). Reduction of mycotoxin exposure and related adverse health effects in these communities can realistically be based on practical low-cost measures only. It needs to be investigated whether a measurable reduction of exposure can be achieved on a practical level in maize subsistence communities.

The traditional maize food preparations in the communities of the Centane magisterial district, South Africa, includes the sorting of maize on the cob (ear) into visibly healthy (good) and visibly fungal infected (mouldy) maize. However, the

resulting good maize can still contain high levels of fumonisin and consumption of this good maize can result in exposures above the PMTDI (Bolger et al., 2001; Kimanya et al., 2008; Shephard et al., 2005; Van der Westhuizen et al., 2008). Therefore the customary maize food preparation practices were assessed and subsequently optimised to reduce the fumonisin levels in the maize under laboratory-controlled conditions. The ultimate goal was to be able to recommend a means of fumonisin reduction, which would be both culturally acceptable and practically implementable, in the subsistence farming communities. This simple method of sorting maize by removal of the infected/damaged kernels and the subsequent washing of the good maize kernels effectively reduced fumonisin contamination of home-grown maize.

Although previous studies achieved reduction of fumonisin in maize by different food preparation procedures, agricultural practices, sorting, mechanical shelling and dehulling, no intervention was implemented (Afolabi et al. 2006; Fandohan et al. 2005; 2006; Kimanya et al. 2009). This study implemented and evaluated the effectiveness of the simple and culturally acceptable intervention method, optimised under laboratory-controlled conditions, to reduce fumonisin exposure in a maize subsistence community. At the baseline phase of the study, participants consumed their customarily prepared porridge twice daily for two consecutive days. They donated a portion of their porridge for fumonisin analyses and completed 24-hour dietary recall questionnaires on the following day. Home-grown maize samples were collected, subsamples were retained for fumonisin analyses, and the remaining maize was pooled, thoroughly mixed and divided into batches. During the intervention phase of the study, participants were trained to apply hand-sorting to the

batches by identifying the infected maize kernels to ensure proper selection for removal and to follow the correct washing procedure. Porridge was prepared from the sorted and washed maize and consumed by the participants twice daily for two consecutive days. The two-step method to reduce fumonisin exposure was evaluated by comparing fumonisin levels in maize and porridge at baseline with the levels following intervention.

The heterogeneous nature of maize contamination means that neither food analysis nor dietary questionnaires alone provide reliable measures of exposure. The simple intervention method reduced fumonisin exposure as assessed by food intake and fumonisin food analysis in a subsistence maize farming community. This part of the study validated the urinary FB₁ biomarker and confirmed the reduction in fumonisin exposure at an individual level. Morning first void urine samples were collected from each participant in the above intervention study on the subsequent days following the consumption of the porridge meals. Urinary FB₁ levels were determined by a newly developed LC-MS method. Fumonisin exposure based on fumonisin levels in the porridge and the amount consumed were compared with the urinary FB₁ levels at baseline and intervention.

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2

Literature Overview

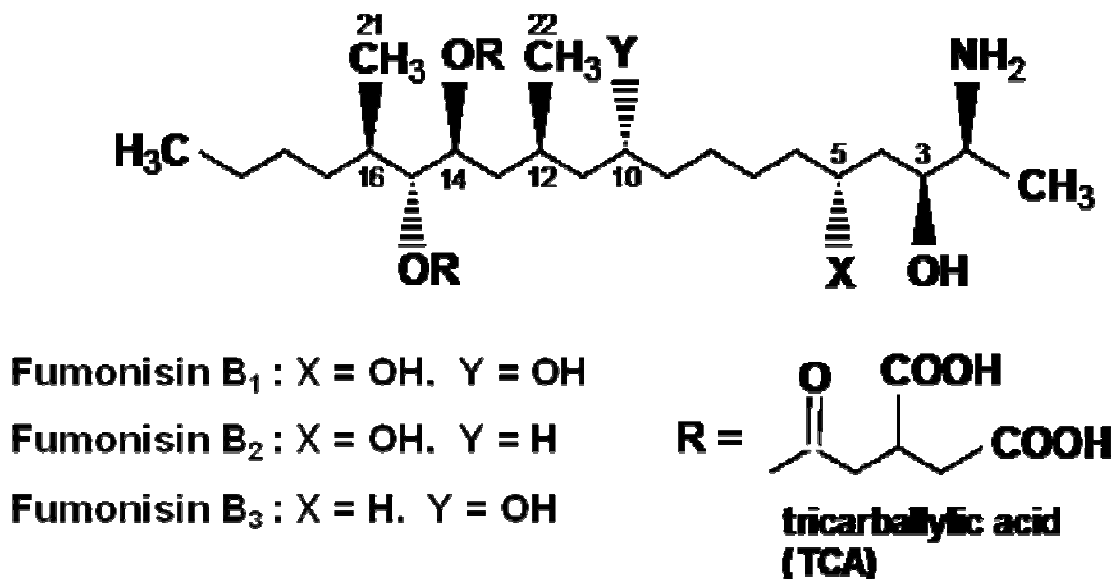
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1. Introduction to fumonisins

Fumonisin are secondary metabolites produced predominantly by *Fusarium verticillioides* (Sacc.) Nirenberg (formerly known as *F. moniliforme* Sheldon) and *F. proliferatum* (Matsushima) Nirenberg (Marasas, 2001). These mycotoxins occur widely around the world in maize (*Zea mays* L.) and maize-based products intended for human consumption (Shephard et al., 1996a). At least 28 fumonisin analogues have been described and categorised into A, B, C, and P series (Rheeder et al., 2002). Fumonisin B₁, B₂ and B₃ (FB₁, FB₂ and FB₃) are the most abundant naturally occurring fumonisins (Figure 1) of which FB₁ is the most significant analogue usually dominating at > 70% of the total fumonisins (FB₁ + FB₂ + FB₃) detected in natural maize samples (Shephard et al. 1996, Rheeder et al., 2002). The other fumonisin series differ from the FB series in that the FA series are acetylated on the amino group at the C-2 position whereas the FB series have a free amine; the FC series

lack the methyl group at the C-1 position and the FP series have a 3-hydroxypyridinium functional group at the C-2 position (Bezuidenhout et al., 1988; Plattner et al., 1992; Musser et al., 1996).



Chapter 2 - Figure 1 The stereochemical structures of fumonisin B₁, B₂ and B₃ (FB₁, FB₂ and FB₃).

Fumonisin are not mutagenic nor genotoxic in primary rat hepatocytes (Norred et al., 1992). However FB₁ exhibits clastogenesis (Ehrlich et al., 2002; Gelderblom et al., 1991; Knasmüller et al., 1997; Mobio et al., 2000) and epigenetic properties in cell cultures. Fumonisin cause various distinct syndromes in different animals, such as leukoencephalomalacia in horses, pulmonary oedema in pigs and neural tube defects in mice (Kellerman et al., 1990; Harrison et al., 1990; Gelineau-van Waes et al., 2005). In addition, FB₁ is hepatocarcinogenic in male BD IX rats and in B6C3F₁ female mice and nephrocarcinogenic in male Fischer 344 rats (Gelderblom et al., 2001; Howard et al., 2001).

High levels of fumonisins have been reported in naturally contaminated maize from areas where high incidences of oesophageal cancer occur, viz., Centane magisterial district, Eastern Cape Province, South Africa; South Carolina, USA; Cixian County of Hebei Province and Linxian County of Henan Province, China, Northern Italy, and Mazandaran and Isfahan Provinces, Iran (Chu and Li 1994; Doko and Visconti, 1994; Shephard et al., 2000; 2002a; Sydenham et al., 1991; Wang et al., 2000; Yoshizawa et al., 1994; Zhang et al., 1997). Fumonisin has also been associated with primary liver cancer in Haimen, Jiangsu Province, China (Ueno et al., 1997). Based on the available data, the International Agency for Research on Cancer has classified FB₁ to be “possibly carcinogenic to humans” (group 2B carcinogen) (IARC, 2002). In addition, the consumption of fumonisin contaminated maize has been reported as one of the risk factors for human neural tube defects (NTD) (Marasas et al., 2004). The co-occurrence of high NTD incidence and consumption of fumonisin contaminated maize has also been reported in various areas, i.e. the Eastern Cape Province of South Africa; the Northern provinces of China and along the Texas-Mexico border in Northern America (Marasas et al., 2004; Missmer et al., 2006).

2. Oesophageal cancer in the Eastern Cape Province of South Africa

The former Transkei region, Eastern Cape Province, South Africa, is one of the areas with the highest incidence rates of oesophageal cancer in the world (Makaula et al., 1996; Rose, 1973; Somdyala et al. 2003a; 2003b). Population-based cancer registry studies have shown that the mean age-standardized incidence rate (ASIR)



Chapter 2 - Figure 2: This map highlights the south eastern (Centane) and north eastern (Bizana) magisterial areas of the former Transkei region, Eastern Cape Province.

for oesophageal cancer were consistently higher in males than females and in the Centane (south eastern) than in the Bizana (north eastern) magisterial district (Figure 2). Earlier studies in the region reported 20-fold higher oesophageal cancer rates in Centane than in Bizana (Rheeder et al., 1992). Even though more recent studies have shown a rising incidence rate in Bizana, Centane has maintained consistently higher oesophageal cancer incidence rates (Makaula et al., 1996; Somdyala et al., 2003a; 2003b). The ASIRs reported recently for men and women of 32.7 and 20.1, respectively, in 8 magisterial districts of the former Transkei region for the period 1998-2002 (Somdyala et al., 2010) were similar to the ASIR for Bizana for the period 1996-2000 (Somdyala et al., 2003b) (Table 1).

Chapter 2 - Table 1 Oesophageal cancer incidence rates (ASIR) in two magisterial districts in the former Transkei region.

Period	ASIR*			
	High incidence of OC		Low incidence of OC	
	Centane		Bizana	
	Males	Females	Males	Females
1955–1959 ^a	54.2	30.3	2.6	1.8
1965–1969 ^b	39.7	16.1	10.5	4.4
1981–1984 ^b	45.0	23.3	19.5	15.0
1985–1990 ^b	55.6	22.1	37.0	11.7
1991–1995 ^c	89.2	32.0	22.8	16.6
1996–2000 ^d	44.8	32.6	31.0	22.7

*ASIR = Age standardized incidence rate/100,000/annum

^aData from Rose et al. 1973

^bData from Makaula et al. 1996

^cData from Somdyala et al. 2003a

^dData from Somdyala et al. 2003b

Chapter 2 - Table 2 Mean total fumonisin levels in maize intended for human consumption from Centane and Bizana.

Season	n	Centane	n	Bizana
		Fumonisin (mg/kg)		Fumonisin (mg/kg)
1985 ^a	12	2.10 (nd–7.90)	12	0.083 (nd–0.55)
1989 ^a	6	1.63 (nd–6.70)	8	0.47 (nd–4.28)
2003 ^b	21	2.18 (nd–8.38)	36	0.36 (nd–6.64)

Values are means (range) or means \pm standard deviation

nd = not detected, < 0.05 mg/kg

^aRheeder et al., 1992

^bRheeder unpublished data

The first study to compare fumonisin levels in home-grown maize in the former Transkei region reported 25-fold higher contamination levels in Centane than in

Bizana (Rheeder et al., 1992) (Table 2). The published studies at that time reported mean oesophageal cancer ASIRs of 20- and 4-fold higher for males and females combined in Centane than in Bizana (Rose, 1973, Rose and Fellingham, 1981). These comparatively high and low oesophageal cancer incidence rates in Centane and Bizana, respectively, corresponded with high and low levels of fumonisins in the home-grown maize from these areas (Rheeder et al., 1992; Marasas, 2001).

3. Fumonisin exposure in subsistence farming communities

The maize cultivated around the world consists of more than 50 different varieties resulting in cobs of different sizes, shapes, colours, and consistencies. Maize is produced to a larger extent than any other grain utilised as staple cereal around the world as it is a high yielding crop, simply processed, easily digested and relatively inexpensive. Africa produced only 7% of the worldwide production of maize in 2009 (FAO, 2010). The worldwide consumption of maize as food in 2009 was only 15% of the total production, whereas Africa imported an additional 28% of their total production for food consumption from countries outside the African continent (IITA, 2010).

Assessing fumonisin exposure by dietary analyses requires a known level of fumonisin contamination in the maize or the maize-based food and the amount of maize or maize-based food consumed daily. The dietary exposure of fumonisin is expressed as the probable daily intake (PDI) of fumonisin per kg body weight (bw):

$$\text{Fumonisin PDI } (\mu\text{g/kg bw/day}) = \frac{\text{Fumonisin in maize } (\mu\text{g/kg}) \times \text{Maize consumed (kg/day)}}{}$$

body weight (kg)

In developed countries maize forms a minor part of the diet as maize intake is estimated at less than 10 g/day in the European Union (EU) and the maize that they consume tends to be of a very high quality (Bolger et al., 2001). Therefore, even if maize were contaminated at extremely high fumonisin levels of 10 mg/kg, their PDI would still be within acceptable limits (Gelderblom et al., 2008) (Table 3). Generally

Chapter 2 - Table 3 The probable daily intake (PDI) of fumonisins (FB) at specific maize intake quantities and different FB contamination levels compared to the group fumonisin provisional maximum tolerable daily intake (PMTDI) of 2 µg/kg body weight/day determined by the Joint FAO/WHO Expert Committee on Food Additives (JECFA).

	Maize Intake (g/day)[†]			
FB (mg/kg)	10	250	460	
0.2	0	0.8	1.5	PDI (µg/kg bw/day)
0.5	0.1	2.1	3.8	
1	0.2	4.2	7.7	
2	0.3	8.3	15.3	
4	0.7	16.7	30.7	
10	1.7	41.7	76.7	

[†]Adult 60 kg

bw = body weight

PDI values in Bold are above the PMTDI

in the lesser developed countries, and more specifically in certain rural areas, maize forms a progressively larger part of the diet. In large parts of Africa maize is a dietary staple consumed almost to the exclusion of all other food commodities (Gelderblom et al., 2008). In some of those rural areas maize is grown and consumed by subsistence farmers and the maize might be contaminated with much higher levels of fumonisin. In contrast to developed countries, in rural communities in South Africa,

maize consumption as high as 460 g per person per day has been reported (Shephard et al., 2007a). Furthermore, subsistence farming communities that consume maize as a staple diet can be exposed to total fumonisin levels of up to 13.8 µg/kg body weight/day (Van der Westhuizen et al., 1999). This is of concern as the Joint FAO/WHO Expert Committee on Food Additives (JECFA) has determined a group provisional maximum tolerable daily intake (PMTDI) for fumonisin B₁, B₂ and B₃, alone or in combination, of 2 µg/kg body weight/day (Bolger et al., 2001). The committee based its decision on a no observed adverse effect level (NOAEL) for nephrotoxicity studies in rodents of 0.2 mg/kg body weight/day and a safety factor of 100. Table 3 illustrates the challenge to keep the PDI of subsistence farmers below the PMTDI determined by JECFA compared to the EU or from the rural areas of South America where consumption is estimated at 10 g and 250 g/person/day, respectively (Shephard et al., 2002b).

4. Biomarkers of fumonisin exposure

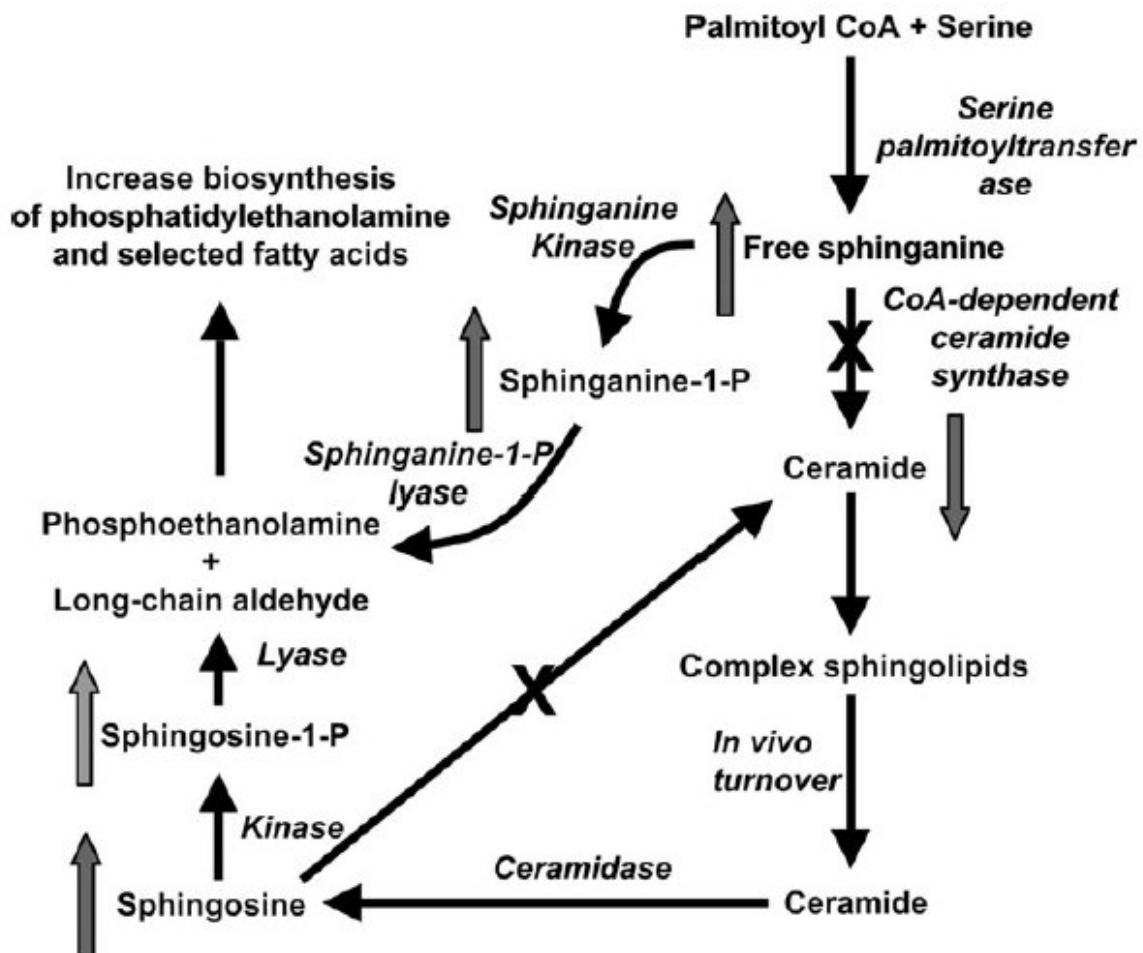
Searching for a biomarker of fumonisin exposure required toxicokinetic (absorption, distribution, biotransformation and excretion) data (Shephard et al., 2007b). A suitable metabolite was sought, but FB₁ did not undergo metabolism when it was subjected to subcellular enzyme fractions in a primary rat hepatocyte culture study (Cawood et al. 1994). Toxicokinetic investigations have shown that FB₁ has a half-life of less than an hour when administered via different routes such as gastric administration, intravenous or intraperitoneally in various animal studies (Shephard et al., 1994; 1995; Fodor et al., 2006). Most of the administered FB₁ was recovered

unaltered and therefore no metabolite of FB₁ suitable as a biomarker for fumonisin exposure was found (Shephard et al., 2007b).

Hair as an alternative biomarker for determining fumonisin exposure has been investigated. FB₁ was detected by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) in hair of rats 4 weeks after administration of a single gavage dose of FB₁ and in vervet monkeys chronically fed diets contaminated with *F. verticillioides* culture material containing relatively high levels of fumonisin (Sewram et al., 2001). Composite human hair samples collected from barber shops in the former Transkei region had detectable levels of FB₁ and very low levels of FB₂ (Sewram et al., 2003). It would thus be possible to utilise hair as a biomarker of fumonisin exposure in humans (Shephard et al., 2007b).

The similarities in the structures of fumonisins and the sphingoid base lipids, sphinganine and sphingosine, led to the investigation of the mechanism of fumonisin action, which revealed that fumonisins inhibit a key enzyme, ceramide synthase, in the *de novo* sphingolipid biosynthetic pathway (Wang et al., 1991) (Figure 3). This inhibition prevents the conversion of sphinganine to dihydroceramide and the reacylation of sphingosine to ceramide (Riley et al., 1994; Wang et al., 1991). The disruption of the sphingolipid biosynthetic pathway elevates sphingoid bases and their 1-phosphate levels and decreases ceramide and more complex sphingolipids, such as sphingomyelin and gangliosides, and their intermediates (Riley et al., 2001; Merrill et al., 2001). Sphingolipids are predominantly found in cellular membranes and are critical for the maintenance of the membrane structure, while complex sphingolipids function as precursors for second messengers and are important in sustaining cellular growth and differentiation (Merrill et al., 2001). This disruption

leads to an elevation of sphinganine levels in cells, and sometimes, to a lesser extent, sphingosine levels, thus resulting in an increase in the sphinganine/sphingosine ratio, as observed in plasma and urine in animal studies (Riley et al., 1993; Shephard et al., 1996b; Van der Westhuizen et al., 2001; Wang et al., 1992). The resultant increase in the sphinganine/sphingosine ratio occurs prior to changes in other biochemical markers of cellular injury, and has thus been proposed as a biomarker of fumonisin exposure (Riley et al., 1994).



Chapter 2 - Figure 3 The *de novo* sphingolipid biosynthetic pathway and degradation pathway illustrating the disruption by FB₁.

(Published in Riley RT, Voss KA Toxicol. Sci. 2006; 92:335-345.)

A preliminary study comparing four male oesophageal cancer patients with female controls from South Africa did not find any significant difference in their serum sphinganine/ sphingosine ratios (Castegnaro et al. 1998). The first investigations on the sphinganine/ sphingosine ratios in plasma and urine from rural populations consuming subsistence maize as their staple diet in Africa were conducted in the Eastern Cape and KwaZulu-Natal Provinces of South Africa, as well as in western Kenya (Van der Westhuizen et al. 1999). This study and subsequent studies conducted in various human populations exposed to different levels of fumonisin have not been able to show that sphinganine or sphingosine levels or the sphinganine/sphingosine ratio can be utilised as a biomarker for fumonisin exposure (Qiu and Liu, 2001; Solfrizzo et al., 2004; Van der Westhuizen et al., 1999).

Although most of the administered FB₁ is excreted almost unchanged in faeces, a small percentage is excreted in urine (Shephard et al., 1994; 1995). However, urine is a more acceptable medium to investigate compared to faeces. Urinary FB₁ has been investigated as a biomarker of exposure and levels of 8 ng FB₁/mL was detected in human urine (Shetty and Bhat et al., 1998).). A recent study in a Mexican population consuming various quantities of maize-based tortillas showed positive correlation between urinary FB₁ and estimates of fumonisin exposure (Gong et al., 2008). Urinary FB₁ levels of 19–248 pg /mL was determined by high performance liquid chromatography coupled to mass spectrometry (HPLC-MS).

Shephard et al. (2007b) have reviewed the biomarkers of fumonisin exposure extensively and subsequent investigations are discussed in the relevant chapters of this thesis.

5. Reduction of fumonisin contamination in subsistence grown maize

In many Sub-Saharan countries, reliant on subsistence maize as a major dietary staple, both maize consumption and maize contamination are high and regulatory mechanisms to control fumonisin levels are either lacking or are not enforced (Gelderblom et al., 2008). Even where regulations on fumonisins in maize are in place, they will have no effect on exposure levels in maize subsistence communities consuming large quantities of home-grown maize daily (Gelderblom et al., 2008; Marasas et al., 2008; Wild and Gong, 2010). These communities are the most vulnerable to the toxic and carcinogenic effects of mycotoxins and therefore intervention methods should be simple, cost effective and aligned with the local customs (Desjardins et al., 2000).

Although previous studies achieved reduction of fumonisin in maize by different food preparation procedures, agricultural practices, sorting, mechanical shelling and dehulling, no intervention was implemented (Afolabi et al., 2006; Fandohan et al., 2005; 2006; Kimanya et al., 2009). An intervention study in Guinean villages resulted in a 60% aflatoxin reduction in groundnuts by introducing primary prevention strategies at postharvest and by introducing the local farmers to readily available materials and local agricultural expertise (Turner et al., 2005). In contrast to aflatoxin, where unsuitable storage practices contribute to increased levels, fumonisin contamination is mainly produced prior to harvesting (Wild and Gong, 2010).

Preharvest insect herbivory, in particular that of the maize stalk borer, damages maize cobs leading to *Fusarium* infection and production of fumonisins (Miller et al., 2001). *Bt* maize has been genetically modified to contain the *cry* genes from *Bacillus thuringiensis*, which upon expression produce insecticidal proteins toxic to Lepidopteran insects, among others the maize stalk borer (Hammond et al., 2004). However, the practice in maize subsistence farming communities is to use the best cobs from the harvest for subsequent planting. Therefore genetically modified maize biotechnology such as *Bt* maize to reduce fumonisin-contamination is not a viable option in these communities due to financial constraints.

Fumonisin contamination of maize is non-homogenous and can be effectively reduced by the removal of visibly infected kernels as demonstrated in a Nigerian study (Afolabi et al., 2006; Whitaker et al. 1998). Fandohan et al., (2005) reported that the processing procedures for traditionally prepared maize meal dishes from Benin reduced fumonisin levels in maize by up to 87% depending on the specific food type. As fumonisin levels are higher in the pericarp of the maize kernel, different mechanical dehulling methods reduced fumonisin contamination by 57–65% (Fandohan et al., 2006; Sydenham et al., 1995). Therefore, sorting, winnowing, washing and dehulling of maize kernels were very effective in achieving these reductions. However, the actual cooking process did not achieve a reduction (Fandohan et al., 2005). This was in contrast to the 23% fumonisin reduction obtained by the traditional cooking process of stiff porridge prepared from South African commercial maize meal (Shephard et al., 2002b). Reduction in fumonisin contamination in subsistence grown maize from Tanzania was also achieved by

selecting specific maize hybrids, reducing plant stress by suitable fertilisers and sorting of maize prior to storage (Kimanya et al., 2009).

6. Conclusion

Fumonisin cause various animal diseases and syndromes and are carcinogenic to rodents. The aetiology of fumonisin in high oesophageal cancer incidence and neural tube defects in humans is still under investigation. However, there is a strong association between consumption of fumonisin-contaminated maize and the incidence of oesophageal cancer. Various biomarkers for fumonisin exposure have been validated in animal studies, of which the sphinganine/sphingosine ratio has been investigated the most extensively. In contrast to animal studies where controlled high doses of fumonisin were administered, most human populations are exposed to varying levels of contamination which might be too low to detect significant differences in the sphingoid bases. Various methods of reducing fumonisin post-harvest in subsistence grown maize have been investigated, but no culturally specific intervention had been conducted in these communities.

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3

**Fumonisin occurrence in
subsistence maize from a
high oesophageal cancer
incidence area**

3.1

Fumonisin contamination and *Fusarium* incidence in maize from Santa Catarina, Brazil

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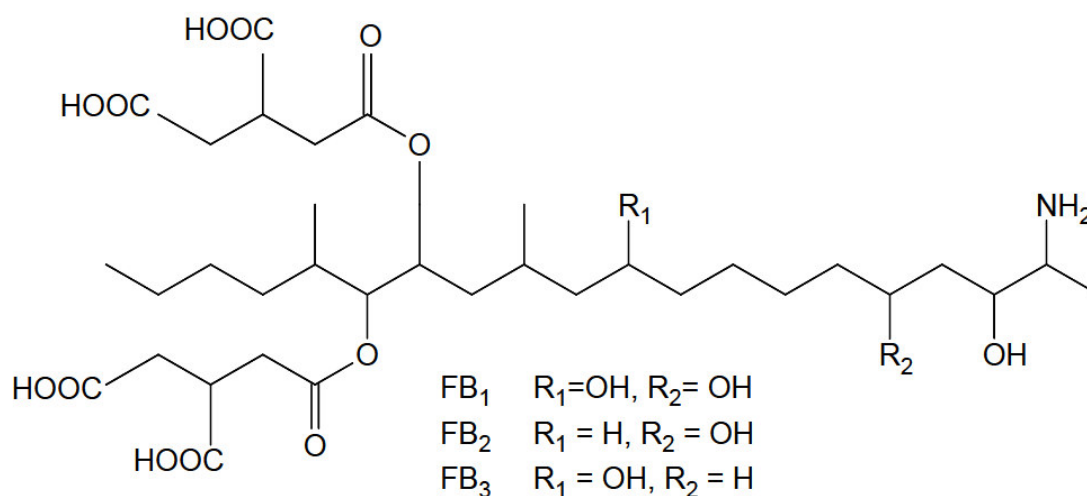
Abstract

In Brazil, the southern region has the highest incidence of oesophageal cancer and also the highest production and consumption of maize (*Zea mays*) products. Maize samples intended for human consumption from the western, northern and southern regions of Santa Catarina State, southern Brazil, had mean total fumonisin B (B₁, B₂ and B₃) levels of 3.2, 3.4 and 1.7 mg/kg, respectively. *Fusarium verticillioides*, the predominant fungus in the maize samples, had a mean incidence (% kernels infected) of 14%, 11% and 18% for the three regions, respectively. Additional maize samples intended for animal feed from the southern region had a mean total fumonisin level of 1.5 mg/kg and a mean *F. verticillioides* incidence of 10%. The fumonisin levels in maize from the State of Santa Catarina, Brazil, were similar to the high levels determined in other high oesophageal cancer incidence regions of the world.

Introduction

Fumonisin, produced predominantly by *Fusarium verticillioides* (Sacc.) Nirenberg (formerly known as *F. moniliforme* Sheldon) and *F. proliferatum* (Matsushima) Nirenberg, occur widely around the world on maize (*Zea mays*) (Shephard et al., 1996). The major naturally occurring fumonisin analogues in maize are fumonisin B₁ (FB₁), B₂ (FB₂) and B₃ (FB₃), see Figure 1.

The contamination of maize with fumonisins is of concern as these mycotoxins cause various animal diseases and occur in maize and maize-based products intended for human consumption (Shephard et al., 1996). In addition, high levels of fumonisins have been found in naturally contaminated maize from areas where high incidences of oesophageal cancer occur, e.g., Centane District, Transkei region of South Africa; Cixian County, Hebei Province, China; and Mazandaran Province, Iran (Chu et al., 1994; Rheeder et al, 1992; Shephard et al., 2000; 2002). Based on current data, the International Agency for Research on Cancer has classified FB₁ to be possibly carcinogenic to humans (class 2B carcinogen) (IARC, 2002).



Chapter 3 - Figure 1 Chemical structures of fumonisin B₁, B₂ and B₃ (FB₁, FB₂ and FB₃).

Brazil is the third largest producer of maize in the world, of which the southern region is the highest producer and consumer of maize-based products. A considerable portion of this maize crop is produced by small farmers and nearly 25% of the harvest is consumed on these farms (Orsi et al., 2000). Santa Catarina, Paraná and

Rio Grande do Sul States, southern Brazil, have the highest incidences of oesophageal cancer in the country, with an age-standardized incidence rate (ASIR) of 18 per 100,000 (INC, 1989). The situation in southern Brazil is similar to other rural areas, where high oesophageal cancer incidence and high maize consumption co-occur (Chu et al., 1994; Rheeder et al., 1992; Shephard et al., 2000). In a survey conducted in Florianópolis in Santa Catarina, Brazil, oesophageal cancer patients accounted for 134 of 2495 total cancer cases registered, mostly originating from the southern and western regions of Santa Catarina. These regions are also the main maize producing areas of Santa Catarina and these populations consume maize-based products as their staple diet (Scaff et al., 1999). The first report on the natural occurrence of fumonisins in maize in Brazil was from feed samples associated with outbreaks of confirmed and suspected mycotoxicoses in various animal species collected from farms in the State of Paraná. These samples were contaminated with mean levels of 8.9 mg/kg FB₁ and 2.8 mg/kg FB₂ (Sydenham et al., 1992). The first report on the occurrence of fumonisins in Brazilian maize-based food products, acquired from markets in Campinas, São Paulo, showed 35 of 72 products to be contaminated with FB₁, with a mean level of 0.4 mg/kg. However, an additional 9 maize meal samples, also acquired from these markets, were all contaminated and showed a much higher mean FB₁ level of 2.3 mg/kg (Machinski et al., 2000). The highest natural fumonisin contamination in maize in Brazil was from freshly harvested samples from the State of São Paulo that had mean levels of 16.4 mg/kg FB₁ and 10.7 mg/kg FB₂ (Orsi et al., 2000). In a further study, maize samples from various cultivars grown in São Paulo State showed mean levels of 5.6 mg/kg FB₁ and 1.9 mg/kg FB₂ (Camargos et al., 2000). Maize samples collected at wholesale markets during the same season in Central, South and Southeast Brazil, had a mean

FB₁ level of 2.2 mg/kg (Vargas et al., 2001). *F. verticillioides* was the predominant *Fusarium* species detected in Brazilian maize in the southeastern State of São Paulo and the southern State of Paraná (Orsi et al., 2000; Sydenham et al., 1992; Almeida et al., 2000; Pozzi et al., 1995; Ono et al., 2002).

The current study was undertaken in the western, northern and southern regions of the State of Santa Catarina, Brazil. Maize samples were collected from these regions during the year 2000 and analyzed for FB₁, FB₂ and FB₃ levels and fungal incidence. In Brazil the southern region has the highest incidence of oesophageal cancer, and this is the first report of high fumonisin levels in maize from Santa Catarina State, southern Brazil.

Materials and methods

Maize samples

Maize samples from the 1999-2000 harvest season were collected during the year 2000 from rural areas of the State of Santa Catarina, Brazil. These samples had been mechanically harvested and shelled, where after the grain was stored in silos. Maize intended for human consumption was collected from mountainous areas in the western (39 samples) and northern (17 samples) regions and from the prairies in the southern region (20 samples), as well as maize intended for animal feed in the southern region (14 samples). The maize samples were sent to the PROMEC Unit, South Africa for fumonisin and mycological analyses.

Analytical methods

Fumonisin analyses

The standards were purified according to the method of Cawood et al. (1991). FB₁, FB₂ and FB₃ levels were determined according to the method of Shephard et al. (2001). Each sample was ground in a laboratory mill to a fine meal and extracted with methanol:water (3:1) by homogenization. An aliquot was applied to a strong anion exchange solid phase extraction cartridge (Varian, Harbor City, CA) and the fumonisins were eluted with 1% acetic acid in methanol. The purified extracts were evaporated to dryness, redissolved in methanol and derivatised with o-phthaldialdehyde. The derivatised extracts were analyzed by reversed-phase high-performance liquid chromatography (HPLC) using an Ultracarb 5 ODS column (Phenomenex, Torrance, CA) and fluorescence detection.

Mycological analyses

Samples were mycologically analyzed for fungal incidence (% kernels infected) using the method of Nelson et al. (1983). Briefly, sub samples of kernels (80–100 g) were surface sterilized for 1 min in 3.5% commercial sodium hypochlorite solution and rinsed twice in sterile water. One hundred kernels (5 kernels/ 90 mm petri-dish) were then transferred to malt extract agar (1.5%) containing novobiocin (150 mg/L) and the agar plates were incubated at 25°C in the dark for 5 to 7 days. All the isolated fungi were recorded and their frequencies determined using a stereo-microscope. *Fusarium* species were identified according to Nelson et al. (1983) and other fungi were identified on the basis of their cultural and morphological characteristics, i.e., *Aspergillus* and *Diplodia* species.

Statistical analysis

The results were statistically analyzed with the Systat 10 software package (SPSS Inc., Chicago, IL) and the correlation of the total fumonisin levels with the *F. verticillioides* incidence with the STATA statistical software package (STATA Corp., College Station, TX).

Results

The mean fumonisin (FB₁, FB₂ and FB₃) levels determined in the maize samples of the different regions are shown in Table 1. FB₁, FB₂ and FB₃ were present in all the maize samples, except for 2 samples in which FB₃ was not detected. The mean level of the total fumonisins in the maize from all three regions in Santa Catarina combined was 2.89 mg/kg (n = 90, range 0.02–18.74 mg/kg), indicating the occurrence of some very high levels (individual results are not shown). In this study 31 of 90 maize samples had FB₁ levels higher than 2 mg/kg and 5 of 90 samples higher than 4 mg/kg, whereas 46 of 90 maize samples had total fumonisin levels higher than 2 mg/kg and 15 of 90 samples higher than 4 mg/kg. In the western, northern and southern regions 24 of 39, 14 of 17 and 8 of 34 samples, respectively, had total fumonisin levels higher than 2 mg/kg. The 14 maize samples intended as animal feed, collected from the southern region, had a mean total fumonisin level of 1.53 mg/kg, whilst the 76 maize samples, intended for human consumption, had a mean total fumonisin level of 2.87 mg/kg (Table 1). There were no statistical differences ($p > 0.05$) in the fumonisin levels between the different regions and the

maize samples intended for human and animal consumption, due in part to large individual variation in the samples as indicated by the standard deviations (Table 1).

Chapter 3.1 - Table 1 Fumonisin levels (mg/kg) in maize samples from the State of Santa Catarina, southern Brazil.

Region	n	FB ₁	FB ₂	FB ₃	Total Fumonisin	
					Mean	Range
West	39	2.06±2.04	0.79±0.80	0.36±0.33	3.21±3.09	0.02–18.74
North	17	2.24±1.01	0.91±0.63	0.30±0.17	3.42±1.75	1.01–7.73
South (HC^a)	20	1.28±2.14	0.35±0.69	0.10±0.17	1.73±2.99	0.14–13.07
Combined (HC)	76	1.89±1.90	0.70±0.76	0.28±0.28	2.87±2.87	0.02–18.74
South (AF^b)	14	1.05±0.85	0.37±0.50	0.11±0.11	1.53±1.36	0.15–4.79

Values are means ± standard deviation

^aHC - Intended for human consumption

^bAF - Intended for animal feed

Differences between the means in maize intended for human consumption from all three regions are not statistically significant ($p > 0.05$)

Differences between the means in animal feed and the maize intended for human consumption from all three regions are not statistically significant ($p > 0.05$)

Whereas the fumonisin contamination of the maize samples was 100%, *Fusarium* species were isolated from 93% of the samples. Even though *F. verticillioides* was the predominant fungus isolated from all three regions (Table 2), the incidence did not correlate with the fumonisin levels in the maize samples ($r = -0.14$, $p > 0.05$). There were no statistical differences ($p > 0.05$) in the mean incidence of *F. verticillioides* in the maize intended for human consumption between the western (14%, range 0–62%), northern (11%, 0–66%) and southern regions (18%, 1–42%), respectively. There was no statistical difference ($p > 0.05$) in the mean incidence of *F. verticillioides* between the maize intended for human consumption (14%, range 0–66%) and the maize intended as animal feed (10%, range 0–36%). There was also

Chapter 3.1 - Table 2 Incidence of fungi in maize samples intended for human consumption (HC) and animal feed (AF) from the State of Santa Catarina, southern Brazil.

Incidence of fungi (% kernels infected)						
Region	n	<i>Fusarium verticillioides</i>	Other <i>Fusarium</i> Species ^a	<i>Aspergillus flavus</i>	Other Species ^b	Total fungi
West	38 ^c	13.5±15.6	1.55±2.85	3.37±4.91	33.2±18.5	51.6±27.2
North	17	11.4±18.7	0.88±2.47	10.2±21.9	20.5±15.1	43.0±34.26
South (HC ^d)	20	17.6±12.5	1.25±2.59	4.05±7.74	36.1±13.7	59.0±23.9
Combined (HC)	75	14.1±15.6	1.32±2.68	5.11±11.8	31.1±17.4	51.6±28.3
South (AF ^e)	14	10.4±12.0	1.71±2.64	8.64±8.34	37.3±13.2	58.1±27.3

Values are means ± standard deviation.

Differences between the means in different regions are not statistically significant ($p > 0.05$).

^aOther *Fusarium* species includes *F. subglutinans* and *F. graminearum*

^bOther species includes *Diplodia maydis* and *D. macrospora*

^cSample received as ground meal. Mycological analysis was performed by dilution plating. *F. verticillioides* 0.9×10^6 colony forming units (cfu)/g, other *Fusarium* species 1.0×10^6 cfu/g, other species 1.0×10^6 cfu/g and total fungi 1.4×10^6 cfu/g.

^dHC - Intended for human consumption.

^eAF - Intended for animal feed

no statistical difference ($p > 0.05$) in the other mycological data obtained from the three regions. *F. subglutinans* was isolated in 16 of 38, 2 of 17 and 11 of 34 samples in the western, northern and southern regions, respectively, with a mean incidence (% kernels infected) of 0.9% (range, 0–12%) for the combined regions. *F. graminearum* was present in only 9 of 90 samples and other *Fusarium* species were present in 6 of 90 samples. *A. flavus* were isolated from 26 of 38, 12 of 17 and 23 of 34 samples in the western, northern and southern regions, respectively, with a mean incidence of 5.7% (range, 0–90%) for the combined regions. *Diplodia* (=

Stenocarpella) maydis and *D. macrospora* were recorded in small numbers of samples (10 of 90 and 12 of 90, respectively) with a range of 0–2% kernels infected.

Discussion

High oesophageal cancer incidence areas in South Africa, China and Iran have been associated with populations consuming high levels of maize heavily contaminated with fumonisin (Chu et al., 1994, Gao et al., 1997; Rheeder et al, 1992; Shephard et al., 2000; 2002). In Brazil, the southern region has the highest incidence of oesophageal cancer (INC, 1989). High production and consumption of maize and maize-based products occur in southern Brazil (Orsi et al., 2000, Scaff et al., 1999). Previous studies that investigated the levels of fumonisin in Brazilian maize were confined almost exclusively to Paraná, southern Brazil and São Paulo, south-eastern Brazil (Orsi et al., 2000; Sydenham et al., 1992; Camargos et al., 2000; Machinski et al., 2000; Ono et al., 1999; 2001; 2002; Hirooka et al., 1996). This study is the first to report fumonisin levels in maize in Santa Catarina State, southern Brazil.

The mean FB₁ level in maize samples from Santa Catarina intended for human consumption was 1.89 mg/kg, which is similar to FB₁ levels in other high esophageal cancer incidence regions, e.g., 1.84 mg/kg in Centane, South Africa in 1989, 2.27 mg/kg in Mazandaran, Iran in 1998 and 2.73 mg/kg in Linxian County, China in 1994 (Chu et al., 1994; Rheeder et al, 1992; Gao et al., 1997). Similar mean FB₁ levels have been reported in other southern Brazilian States. During the 1997/1998 season maize from central, southern and south-eastern Brazil had a mean FB₁ level of 2.2

mg/kg in 214 samples and in the 1995/1996 season from central-western Paraná a mean level of 2.4 mg/kg in 86 samples (Vargas et al., 2001, Ono et al., 1999). The first investigation on fumonisin contamination in maize-based food products conducted in São Paulo revealed a mean FB₁ level of 0.4 mg/kg, whereas maize meal samples collected from the same markets had a mean FB₁ level of 2.3 mg/kg (Machinski et al., 2000).

The mean total fumonisin level in maize intended for human consumption was 2.87 mg/kg, almost double the 1.53 mg/kg level in the maize intended as animal feed. However, the difference was not statistically significant, due in part to individual variation in the samples as indicated by the standard deviation. Based on this mean total fumonisin contamination of 2.87 mg/kg and the assumption that a 70 kg person from the rural area in Brazil consumes 11 to 39 g of dry maize per day (Machinski et al., 2000), the probable daily intake (PDI) of fumonisins by this population was up to 1.6 µg/kg body weight/day. This is double the tolerable daily intake (TDI) of 0.8 µg/kg body weight/day, which was based on a NOEL (no observed effect level) of 25 mg FB₁ /kg diet in rats and a safety factor of 1000 for carcinogenicity (Gelderblom et al., 1996). However, the Joint FAO/WHO Expert Committee (JECFA) on Food Additives recommended a provisional maximum tolerable daily intake (PMTDI) for total fumonisins of 2 µg/kg body weight/day based on a NOEL of 0.2 mg/kg weight/day and a safety factor of 100 (Bolger et al., 2001). Considering this recommendation the exposure of the rural population of Santa Catarina would be below the level proposed by JECFA.

Similar to previous Brazilian investigations on the mycoflora of maize, *F. verticillioides* was the predominant fungus isolated in this study (Orsi et al., 2000, Sydenham et al., 1992; Almeida et al., 2000; Pozzi et al., 1995; Ono et al., 2002). However, the incidence of *F. verticillioides* was not significantly correlated with the fumonisin levels in the maize as reported in other South African maize studies (Rheeder et al., 1992; 1995). In contrast to these studies, a correlation was observed in investigations on maize conducted in Argentina, South Africa and the United States of America (Sydenham et al., 1991; 1993). The presence or absence of significant positive correlations might be attributed to various factors that influence the production of fumonisins by *F. verticillioides*, e.g., the maize hybrid, the abiotic stress on the host plant and symptomless systemic infection (Rheeder et al., 1992; Orsi et al., 2000; Bacon et al., 2001). Even though *F. subglutinans* was isolated in 29 of 89 samples, the mean incidence was only 0.9% and that for *F. graminearum* only 0.2%, and neither of these two *Fusarium* species produce fumonisins (Rheeder et al., 2002). Therefore, compared to *F. verticillioides* the incidence of the other *Fusarium* species in this study was insignificant. The second most abundant genus of fungi isolated from the maize was *Aspergillus* (5.7%). Co-contamination with aflatoxin and fumonisin has previously been reported in Brazilian maize from central, southern and south-eastern Brazil, specifically in the states of Paraná and São Paulo (Vargas et al., 2001; Almeida et al., 2000; Ono et al., 2002). Further investigation is required into the seasonal variation of the chemical and mycological data in the maize of Santa Catarina, Brazil, as differences may be expected due to changing environmental conditions such as annual rainfall, temperature and insect infestation. Additional epidemiological data on ASIR of oesophageal cancer in Santa Catarina are also urgently required. The high level of fumonisin contamination in maize

intended for human consumption in yet another region where the incidence of oesophageal cancer is high, is a further indicator for an association between the consumption of maize contaminated with fumonisins and oesophageal cancer.

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4

**The effect of fumonisin B₁ on
sphingolipid biosynthesis
in rat liver nodules**

4.1

Disruption of sphingolipid biosynthesis in hepatocyte nodules: selective proliferative stimulus induced by fumonisin B₁

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Abstract

In order to investigate the role of sphingolipid disruption in the cancer promoting potential of fumonisin B₁ (FB₁) in the development of hepatocyte nodules, male Fischer 344 rats were subjected to cancer initiation (FB₁ containing diet or diethylnitrosamine [DEN] by intraperitoneal injection) and promotion (2-acetylaminofluorene with partial hepatectomy [2-AAF/PH]) treatments followed by a secondary FB₁ dietary regimen. Sphinganine and sphingosine levels were measured by high performance liquid chromatography in control, surrounding and nodular liver tissues of the rats. The disruption of sphingolipid biosynthesis by the secondary FB₁ treatment in the control rats was significantly ($p<0.05$) enhanced by the 2-AAF/PH cancer promotion treatment. The nodular and surrounding sphinganine levels returned to baseline following FB₁ initiation and 2-AAF/PH promotion. When comparing the groups subjected to the secondary FB₁ treatment, the initiation effected by FB₁ was less ($p<0.01$) sensitive to the accumulation of sphinganine in the nodular and surrounding tissues than DEN initiation and the 2-AAF/PH control treatment. In contrast, the sphingosine level of FB₁ initiation was marginally increased in the nodules compared to the surrounding liver after 2-AAF/PH promotion and significantly ($p<0.05$) higher with the secondary FB₁ treatment. Although, the FB₁ -induced hepatocyte nodules were not resistant to the disruption of sphingolipid biosynthesis, the nodular sphingosine levels were increased and might provide a selective growth stimulus possibly induced by bio-active sphingoid intermediates such as sphingosine 1-phosphate.

Introduction

Fumonisin B₁ (FB₁) is the major mycotoxin predominantly produced by *Fusarium verticillioides* occurring ubiquitously on corn (Shephard et al., 1996). Ingestion of fumonisin-contaminated feed results in various animal diseases (Shephard, 2001). High incidences of human esophageal cancer (Rheeder et al., 1992; Chu and Li, 1994; Yoshizawa et al., 1994) and liver cancer (Ueno et al., 1997) have been associated with the consumption of fumonisin-contaminated corn. Fumonisin is not mutagenic (Gelderblom et al., 1991; Knasmüller et al., 1997) nor genotoxic in primary rat hepatocytes (Norred et al., 1992), however FB₁ exhibits clastogenesis (Ehrlich et al., 2002) and epigenetic properties (Mobio et al., 2000) in cell cultures. FB₁ is hepatocarcinogenic in male BD IX rats (Gelderblom et al., 1991; 2001) and in B6C3F₁ female mice and nephrocarcinogenic in male Fischer 344 rats (Howard et al., 2001).

FB₁ inhibits ceramide synthase, a key enzyme in de novo sphingolipid biosynthesis, preventing the conversion of sphinganine to dihydroceramide and the reacylation of sphingosine to ceramide (Riley et al., 1994; Wang et al., 1991). The disruption of the sphingolipid biosynthetic pathway elevates sphingoid bases and their 1-phosphate levels and decreases ceramide and more complex sphingolipids, such as sphingomyelin and gangliosides, and their intermediates (Riley et al., 2001; Merrill et al., 2001). However, an increase in the sphingoid bases can only occur once the capacity of sphingosine kinase to metabolize these bases to their 1-phosphates has been exceeded (Riley et al., 2001). Sphingolipids are predominantly found in cellular membranes and are critical for the maintenance of the membrane structure, while

complex sphingolipids function as precursors for second messengers and are important in sustaining cellular growth and differentiation (Merrill et al., 2001). FB_1 inhibits cell proliferation in various cell culture systems as well as in rat liver and kidney (Gelderblom et al., 1996; Riley et al., 2001; Yoo et al., 1992). FB_1 -induced disruption of sphingolipid biosynthesis can either induce or prevent apoptosis, depending on the cell type and the relative amounts of the bio-active sphingolipid molecules generated (Desai et al., 2002; Tolleson et al., 1996). The impairment of apoptotic pathways during liver cancer promotion results in an imbalance between cell death and proliferation and thus the outgrowth of hepatocyte nodules in the presence of a promoter (Schulte-Hermann et al., 1993). In this regard cells with decreased ceramide and increased sphingosine 1-phosphate levels might be selected to survive and proliferate, provided that increased sphingoid bases are not growth inhibitory in these cells (Riley et al., 2001). Hence, the disruption of sphingolipid biosynthesis has been implicated in the carcinogenic activity of FB_1 (Riley et al., 2001; Voss et al., 2002).

Cancer initiation by chemicals in rat liver is generally characterized by the appearance of phenotypically altered resistant hepatocytes (Solt et al., 1980). These resistant hepatocytes escape the mitoinhibitory effects of FB_1 on normal hepatocyte growth and selectively proliferate into hepatocyte nodules (Gelderblom et al., 1995; 2001). The exact mechanism involved in the selection of initiated cells by FB_1 is unknown. The purpose of this study was to determine whether hepatocyte nodules are resistant to the inhibitory effect of FB_1 on ceramide synthase, resulting in a growth differential which could selectively stimulate their outgrowth.

Materials and methods

Chemicals

FB₁ was purified as described previously by Cawood et al. (1991). Diethylnitrosamine (DEN), 2-acetylaminofluorene (2-AAF), sphinganine and sphingosine were obtained from Sigma Chemical Company (St. Louis, MO, USA). C20-sphinganine was a generous gift from Prof. A. H. Merrill Jr. All other chemicals and solvents were analytical grade from Merck (Darmstadt, Germany).

Animals

Male Fischer (F344) rats were bred and maintained on the AIN76 diet (AIN, 1977) at the Primate Unit of the MRC Diabetes Research Group in a controlled environment of 23-25°C and 12 h light/dark cycles. During the experimental period the rats were caged individually with normal access to feed and water. The experimental protocol was approved by the Ethics Committee for Research on Animals of the Medical Research Council, Tygerberg, South Africa.

Experimental procedures

Experimental male Fischer 344 rat (150-200 g) groups were subjected to various cancer initiation and promotion regimens, whereas control rat groups were subjected only to the promotion regimens (Table 1). Initiation treatment consisted of either a 3-week FB₁ dietary treatment (500 mg/kg feed) or a single intraperitoneal (i.p.) injection of diethylnitrosamine (DEN, 200 mg/kg body weight). Promotion treatment followed two weeks after the initiation treatment and consisted of 2-acetylaminofluorene (2-AAF, 20 mg /kg body

Chapter 4.1 - Table 1 Treatment protocol of the control and experimental rat groups for studying whether hepatocyte nodules are resistant to the inhibitory effect of FB₁ on ceramide synthase.

Group number and Treatment code		Initiation ^a 21 days	Recovery 14 days	Promotion ^b 4 days	Recovery 14 days	Secondary treatment 4 days
Control groups						
1	Control	Control diet	Control diet	-	Control diet	Control diet
2	Control/FB₁	Control diet	Control diet	-	Control diet	250 mg FB ₁ /kg diet
3	2-AAF/PH	Control diet	Control diet	2-AAF/PH	Control diet	Control diet
4	2-AAF/PH/FB₁	Control diet	Control diet	2-AAF/PH	Control diet	250 mg FB ₁ /kg diet
Experimental groups						
5	FB₁/2-AAF/PH	500 mg FB ₁ /kg diet	Control diet	2-AAF/PH	Control diet	Control diet
6	FB₁/2-AAF/PH/FB₁	500 mg FB ₁ /kg diet	Control diet	2-AAF/PH	Control diet	250 mg FB ₁ /kg diet
7	DEN/2-AAF/PH	DEN/Control diet	Control diet	2-AAF/PH	Control diet	Control diet
8	DEN/2-AAF/PH/FB₁	DEN/Control diet	Control diet	2-AAF/PH	Control diet	250 mg FB ₁ /kg diet

^aInitiation treatment consisted of either a 3-week FB₁ (500 mg/kg) dietary treatment (groups 5 and 6) or a single i.p. injection (200 mg/kg) of diethylnitrosamine (DEN, groups 7 and 8).

^bPromotion treatment consisted of 2-acetylaminofluorene (2-AAF, 20 mg/kg) gavage doses on three consecutive days followed by partial hepatectomy (PH).

weight) gavage doses on three consecutive days followed by partial hepatectomy (PH) (Semple-Roberts et al., 1987). The rats were anaesthetized with 2-3% fluothane (95% O₂) and received 5% glucose supplementation in their drinking water for 12 hours post-operative. After initiation and promotion all the rats received control diets for a two-week recovery period. Subsequently, 50% of all the groups were subjected to a secondary two-week FB₁ dietary treatment (250 mg/kg feed). At the end of the experimental period all the rats were sacrificed (sagatal anaesthesia) and the macroscopically distinguishable encapsulated hyperplastic nodules were separated by scooping the nodules from the surrounding liver tissue. The nodular, surrounding and control liver tissues were collected, frozen on dry ice and stored at -80°C.

Sphingolipid analyses

Homogenized liver extracts were prepared from all the liver tissues in phosphate buffer (Van der Westhuizen et al., 2001a) and the levels of the sphingolipid bases, sphinganine and sphingosine, quantified by reversed-phase HPLC as fluorescent derivatives (Van der Westhuizen et al., 2001b). Protein content of the liver extracts was determined by a modified Lowry method (Markwell et al., 1978).

Statistical analysis

The data were tested for normality, using the Kolmogorov-Smirnov test, as well as for equality of variances. T-tests were used to test for group differences (2 groups), using the Pooled Method when variances were equal, and the Satterthwaite Method when variances were unequal.

Results

Control treatments

The liver sphinganine ($p < 0.001$) and sphingosine ($p < 0.05$) levels, as well as the sphinganine/ sphingosine ratio ($p < 0.01$), were significantly increased in rats subjected to the secondary FB_1 treatment compared to the baseline levels of the untreated control rats (Table 2). Although both the sphinganine and sphingosine levels were significantly ($p < 0.05$) increased in the liver of the rats subjected to the 2-AAF/PH promotion regimen, the sphinganine/ sphingosine ratio was similar to the baseline ratio. The sphingosine level in the 2-AAF/PH treated rats was similar to the rats subjected to the secondary FB_1 treatment. The secondary FB_1 treatment significantly enhanced the accumulation of sphinganine ($p < 0.05$) and the sphinganine/sphingosine ratio ($p < 0.01$) induced by the 2-AAF/PH promoting regimen.

Initiation protocols using FB_1 and DEN regimens

(i) Feeding the control diet during the secondary treatment period

Hepatocyte nodules were observed in the livers of all the rats, which were subjected to either FB_1 or DEN initiation treatment prior to the 2-AAF/PH promotion regimen as reported previously (Gelderblom et al., 1992; 1996). Histological features of the nodules have been described previously (Gelderblom et al., 2002). The nodules were scattered randomly throughout the liver and sharply demarcated from the surrounding liver and showed increased mitotic figures, mixed eosinophilic and clear cell changes. Oval cells were observed forming a rim around the nodules (Figure 1).

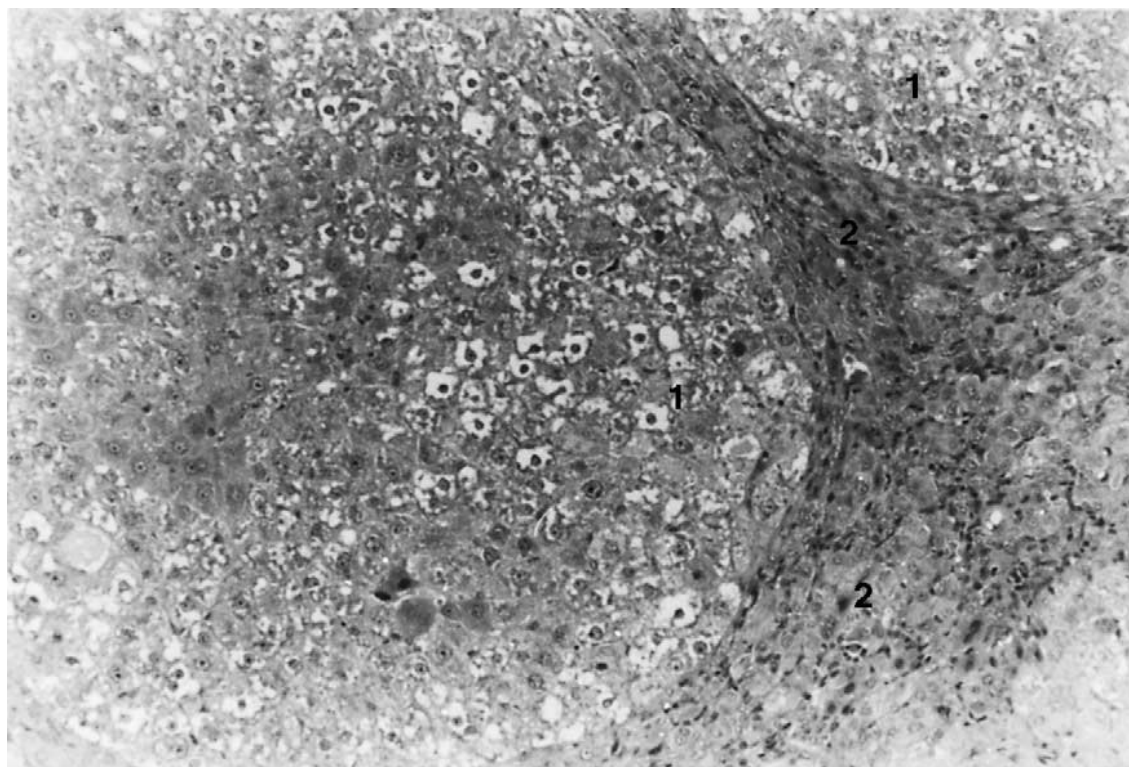
Chapter 4.1 - Table 2 Sphinganine (Sa) and sphingosine (So) levels in rat liver of the different control and experimental groups in control, surrounding and nodular tissues^a

Group number		(n)	Sphinganine	Sphingosine	Sa/So
Treatment regimen			(pmol/mg protein)	(pmol/mg protein)	Ratio
Control					
1	Control	4	1.13 ± 0.15 (1.03–1.35) a	14.7 ± 4.32 (9.57–19.2) a	0.08 ± 0.02 (0.06–0.11) a
2	Control/ FB ₁	4	16.2 ± 2.04 (14.4–18.6) B	27.2 ± 6.75 (17.2–31.6) b	0.63 ± 0.17 (0.46–0.86) B
3	2-AAF/PH	4	1.91 ± 0.46 (1.29–2.39) b	28.3 ± 7.34 (17.9–34.1) b	0.07 ± 0.03 (0.05–0.11) a
4	2-AAF/PH/ FB ₁	6	46.3 ± 25.7 (20.3–90.0) B	36.8 ± 20.3 (11.8–59.0) b	1.38 ± 0.39 (0.78–1.79) B
Surrounding					
5	FB ₁ /2-AAF/PH	4	1.22 ± 0.30 (1.01–1.65) a	20.5 ± 6.32 (11.7–26.6) a	0.06 ± 0.02 (0.04–0.09) a
6	FB ₁ /2-AAF/PH/ FB ₁	4	13.0 ± 3.13 (9.00–16.1) B	32.7 ± 4.49 (28.8–38.9) B	0.39 ± 0.06 (0.31–0.45) B
7	DEN/2-AAF/PH	6	1.11 ± 0.64 (0.59–2.11) a	17.0 ± 10.4 (5.80–32.7) a	0.08 ± 0.03 (0.04–0.12) a
8	DEN/2-AAF/PH/ FB ₁	4	28.6 ± 7.44 (19.4–36.6) B	48.4 ± 17.4 (25.2–67.2) b	0.62 ± 0.12 (0.50–0.77) B
Nodules					
5	FB ₁ /2-AAF/PH	4	2.59 ± 1.33 (1.29–3.98) a	36.8 ± 13.9 (25.7–55.3) b	0.06 ± 0.03 (0.03–0.09) a
6	FB ₁ /2-AAF/PH/ FB ₁	4	15.4 ± 6.58 (9.55–24.4) b	47.2 ± 10.8 (33.3–57.0) B	0.32 ± 0.10 (0.22–0.45) b
7	DEN/2-AAF/PH	5	1.15 ± 0.34 (0.60–1.51) a	21.8 ± 10.4 (9.82–34.6) a	0.06 ± 0.02 (0.04–0.07) a
8	DEN/2-AAF/PH/ FB ₁	6	27.6 ± 8.09 (16.8–38.5) B	50.4 ± 10.5 (38.1–65.3) B	0.55 ± 0.12 (0.37–0.68) B

^aValues represent means standard deviation with the range in brackets.

Values in a column followed by the same letter are not significantly different from the control, if the letter differs then p<0.05, if the cases differ then p<0.01.

The DEN cancer initiation regimen followed by the 2-AAF/PH promotion did not significantly affect either the sphinganine or sphingosine levels in both the nodular and surrounding tissues compared to the baseline levels. The FB₁ cancer initiation regimen did also not significantly affect the sphingoid bases in either the nodular or surrounding tissues as all the levels returned to baseline levels, except for the nodular sphingosine level which was significantly ($p<0.05$) increased above the baseline level.



Chapter 4 - Figure 1: Hepatocyte nodules in a rat from experimental group 6. Note the hepatocyte nodules (1) and proliferating oval cells in the surrounding tissue (2) (H&E×100).

(ii) Feeding the FB₁-containing diet during the secondary treatment period

The FB₁ cancer initiation treatment significantly increased the sphinganine levels to a similar extent in the surrounding ($p<0.01$) and nodular ($p<0.05$) tissues when

compared to the rats that received a control diet during the secondary treatment period. The sphinganine levels in the nodular and surrounding tissues were similar to the control rats treated with the secondary FB₁, but were significantly ($p<0.05$) lower than the control rats subjected to the combined 2-AAF/PH promotion and secondary FB₁ regimens. The nodular sphingosine level was significantly ($p<0.05$) higher compared to the surrounding tissue, but was similar to the control rats subjected to the combined 2-AAF/PH promotion and secondary FB₁ regimens. The sphinganine/sphingosine ratios in both the surrounding ($p<0.001$) and nodular ($p<0.05$) tissues were significantly increased above the baseline ratio, but were significantly lower ($p<0.05$) than the ratios observed in both the control and 2-AAF/PH groups subjected to the secondary FB₁ treatment. When DEN was used as cancer initiator the sphinganine and sphingosine levels were similarly increased in both the surrounding and nodular tissues compared to the rats fed a control diet during the secondary treatment period. The sphinganine levels in the surrounding ($p<0.01$) and nodular ($p<0.05$) tissues were significantly higher than when FB₁ was used as a cancer initiator. Both the surrounding and nodular sphinganine levels were significantly increased ($p<0.01$) over the baseline level and markedly (not significantly) lower than the 2-AAF/PH treated rats subjected to the secondary FB₁ treatment. A similar effect was noticed for the sphingosine level, except that the sphingosine levels were markedly higher in comparison to the 2-AAF/PH rats, subjected to the secondary treatment. The sphinganine/sphingosine ratio was similar in the nodular and surrounding tissues and in the control rats subjected to the secondary FB₁ treatment, but was significantly ($p<0.01$) lower than the 2-AAF/PH subjected to the secondary FB₁ treatment.

Discussion

In normal regenerating liver disruption of sphingolipid biosynthesis induced by FB₁ enhanced the accumulation of sphinganine (Li et al., 2000). In the present study the 2-AAF/PH promotion regimen significantly sensitized the liver to the accumulation of sphinganine by the two-week secondary FB₁ treatment. Additionally, the significant increase in sphingosine, four weeks post 2-AAF/PH treatment, could have enhanced the sphingosine 1-phosphate levels and led to stimulation of cell proliferation and suppression of apoptosis (Desai et al., 2002). As in previous studies, hepatocyte nodules developed in the livers of all the rats subjected to the cancer initiation (FB₁ or DEN) treatment followed by the 2-AAF/PH promotion regimen (Gelderblom et al., 1992; 1996). In the absence of the secondary FB₁ treatment, the sphinganine levels returned to baseline in both the nodules and surrounding tissue six weeks after cessation of FB₁ initiation treatment followed by the 2-AAF/PH promoting regimen. This reversibility of ceramide synthase inhibition was also apparent upon removal of FB₁-contaminated diet in animal studies or when FB₁ is removed from the media in primary as well as transformed cell culture systems (Enongene et al., 2002; Gelderblom et al., 1995; Wang et al., 1999; Yoo et al., 1992). In the presence of the secondary FB₁ treatment, the sphinganine level was enhanced to a similar extent in nodular and surrounding liver, comparable with the control rats treated with the secondary FB₁. However, in the absence of the secondary FB₁ treatment, the nodular sphingosine in the FB₁ initiated rats did not return to baseline level, but remained increased similar to the control group subjected to both the 2-AAF/PH and the secondary FB₁ regimens. The increased nodular sphingosine might be attributed to an increase in cell proliferation induced by the 2-AAF/PH promotion regimen. In

the presence of the secondary FB₁ treatment, the sphingosine level was selectively further increased above the surrounding tissue, especially in the FB₁-induced initiated cell population. It would appear that nodules induced by FB₁ are sensitized to accumulate sphingosine, which could selectively support cell proliferation of initiated cells through the production of sphingosine 1-phosphate.

When utilising DEN as the cancer initiator model, in the absence of the secondary FB₁ treatment, sphingolipid biosynthesis was not disrupted in the nodular or surrounding tissue. However, DEN initiation, followed by the secondary FB₁ treatment, disrupted sphingolipid biosynthesis significantly in the nodules and surrounding liver tissue. Both the nodular and surrounding sphingosine were significantly increased to a similar level as in the FB₁-induced nodules subjected to the secondary FB₁ treatment. It would appear that DEN sensitized nodules and surrounding liver treated with the secondary FB₁ regimen, accumulated sphinganine to a higher extend than FB₁-initiated liver. However, the sphinganine level was still lower than in the control rats subjected to the combined 2-AAF/PH promotion and secondary FB₁ regimen. The increased sensitivity in DEN rats towards the disruption of sphingolipid metabolism by FB₁, implies that FB₁ pre-treatment rendered the liver more resistant to the accumulation of sphinganine, but not sphingosine, which tended to selectively accumulate in the FB₁-induced hepatocyte nodules. This resulted in a significantly lower sphinganine/sphingosine ratio in the FB₁-induced nodules compared to the DEN-induced nodules.

The present study confirmed that normal proliferating hepatocytes are more sensitive to the disruption of sphingolipid biosynthesis by FB₁ than quiescent hepatocytes. The

inhibitory effect of FB₁ on ceramide synthase was reversible in hepatocyte nodules, although an apparent delayed effect on the reversal of sphingosine was observed. The FB₁-induced hepatocyte nodules were not resistant to the disruption of sphingolipid biosynthesis, implying that it might not be a major growth stimulus in their outgrowth. However, the delayed recovery effect of the sphingosine levels in the FB₁-induced nodules compared to the surrounding tissue, and the sensitization of sphingosine accumulation in the nodules upon subsequent FB₁ exposure, could provide a selective growth stimulus resulting in their selective outgrowth.

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5

**Sphingoid base levels in humans
consuming subsistence maize
contaminated with fumonisins**

5.1

Sphingoid Base Levels in Humans Consuming Fumonisin Contaminated Maize from Low and High Oesophageal Cancer Incidence Areas: A Cross Sectional Study

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Abstract

High oesophageal cancer incidence rates have been associated with the consumption of fumonisin contaminated maize in the former Transkei region, Eastern Cape Province, South Africa. In this cross sectional study plasma and urine of male and female participants from Centane magisterial area (highest incidence area) in 1997 and Bizana (lower incidence area) in 2000, were analysed for the sphingoid bases, sphinganine and sphingosine. Good home-grown and visibly mouldy maize samples were analysed for fumonisin B₁, B₂ and B₃. Both the mean plasma female ($p=0.01$) and male ($p>0.05$) sphinganine, as well as the sphingosine levels, were higher in Centane than in Bizana resulting in higher mean sphinganine/sphingosine ratios. The mean male and female urinary sphingosine levels were significantly higher ($p<0.0001$) in Centane than in Bizana; whereas the mean female ratios were significantly lower ($p<0.05$) in Centane than in Bizana. The mean urinary sphingosine levels in the male participants were significantly lower than the female participants within both the magisterial areas. Based on the mean total fumonisin levels in good home-grown maize collected from Centane in 1997, 2000 and Bizana in 2000, the estimated fumonisin exposure was 4.4, 6.7 and 5.8 $\mu\text{g/kg}$ body weight/day, respectively, exceeding the maximum tolerable daily intake for total fumonisins determined by JECFA by two to three-fold.

Introduction

Fumonisin s are mycotoxins produced predominantly by *Fusarium verticillioides* (Sacc.) Nirenberg (formerly known as *F. moniliforme* Sheldon) and *F. proliferatum* (Matsushima) Nirenberg (Marasas, 2001) and the major naturally occurring analogues are fumonisin B₁ (FB₁), B₂ (FB₂) and B₃ (FB₃) (Shephard et al., 1996a). Fumonisin s cause distinct syndromes in different animals such as leukoencephalomalacia in horses, pulmonary oedema in pigs and neural tube defects in mice (Kellerman et al., 1990; Harrison et al., 1990; Sadler et al., 2002). Fumonisin s are not mutagenic (Gelderblom et al., 1991; Knasmüller et al., 1997) nor genotoxic in primary rat hepatocytes (Norred et al., 1992), however FB₁ exhibits clastogenesis (Ehrlich et al., 2002), epigenetic properties (Mobio et al., 2000) and other DNA related damage in cell cultures (De Lorenzi et al., 2005, Galvano et al., 2002). FB₁ is hepatocarcinogenic in male BD IX rats (Gelderblom et al., 2001) and B6C3F₁ female mice and nephrocarcinogenic in male Fischer 344 rats (Howard et al., 2001). As fumonisin s occur widely around the world in maize and maize-based products intended for human consumption there are health implications (Shephard et al., 1996a; Thiel et al., 1992). The Centane magisterial area, former Transkei region of the Eastern Cape Province, South Africa, is one of the areas with the highest incidence of oesophageal cancer in the world, whereas the Bizana magisterial area has comparatively low oesophageal cancer incidence rates (Makaula et al., 1996; Somdyala et al., 2003). These comparatively high and low oesophageal cancer incidence rates in Centane and Bizana, respectively, corresponded with high and low levels of fumonisin s in the home-grown maize from these areas (Rheeder et al., 1992; Shephard et al., 2007). High levels of fumonisin s have also been found in naturally contaminated maize from other areas where high incidences of oesophageal cancer occur, viz., Charleston, SC, USA; Cixian County, Hebei

Province, China; Northern Italy and Santa Catarina, Southern Brazil (Chu and Li, 1994; Doko and Visconti, 1994; Sun et al., 2007; Sydenham et al., 1991; Van der Westhuizen et al., 2003).

Ueno et al. (1997) hypothesized that in high risk areas for primary liver cancer consumption of maize with high levels of fumonisin and trichothecenes promote hepatocarcinogenesis initiated by aflatoxin B or hepatitis B virus. Marasas et al. (2004) proposed that fumonisins are potential risk factors for birth defects arising from neural crest cells because of their interference with folate utilization. A recent study along the Texas-Mexican border confirmed that fumonisin exposure increased the risk for neural tube defects (Missmer et al., 2006). In an evaluation the International Agency for Research on Cancer (IARC) declared fumonisin B1 to be a group 2B carcinogen (IARC, 2002). A risk assessment by the Joint FAO/WHO Expert Committee on Food Additives determined a group provisional maximum tolerable daily intake for total fumonisin of 2 µg/kg body weight/day (Bolger et al. 2001).

Due to similarities in the structures of fumonisins and the sphingoid base lipids, sphinganine and sphingosine, investigation into the mechanism of action has revealed that fumonisins inhibit a key enzyme, ceramide synthase, in the de novo sphingolipid biosynthetic pathway (Wang et al., 1991). This inhibition disrupts sphingoid metabolism resulting in elevated sphingoid bases and their 1-phosphate levels and decreased ceramide and more complex sphingolipids and their intermediates (Riley et al., 2001; Merrill et al., 2001). This disruption leads to an elevation of sphinganine levels in cells, and sometimes, to a lesser extent,

sphingosine levels, thus resulting in an increase in the sphinganine/sphingosine ratio, as observed in plasma and urine in animal studies (Riley et al., 1994; Shephard et al., 1996b; Van der Westhuizen et al., 2001; Wang et al., 1992). As the increase in the sphinganine/ sphingosine ratio due to fumonisin exposure was observed before other biochemical markers of cellular injury, the sphinganine/sphingosine ratio was proposed as a biomarker for fumonisin exposure (Riley et al., 1993).

The first studies in Africa on sphinganine/sphingosine ratios in plasma and urine from rural populations consuming home-grown maize as their staple diet were conducted in two coastal provinces of South Africa and in western Kenya (Van der Westhuizen et al., 1999). The sphinganine/sphingosine ratio seemed not sensitive enough to act as a biomarker in humans even at the highest estimated fumonisin exposure levels of 47 µg/kg body weight/day assuming a 70 kg adult consumes 460 g maize/day (Van der Westhuizen et al., 1999; Shephard et al., 2002). A preliminary study comparing four male oesophageal cancer patients with female controls did not find any significant difference in their serum sphinganine/sphingosine ratios (Castegnaro et al., 1998). No significant association was found between sphingoid base levels and their ratio and the level of risk for oesophageal squamous cell carcinoma in a study conducted in a high oesophageal cancer incidence region (Linxian) in China (Abnet et al., 2001a). An exposure study conducted in Henan Province in China reported significantly higher urinary sphinganine/sphingosine ratios in males only when the mean dietary consumption levels exceeded 110 µg FB₁/kg body weight/day (Qiu and Liu, 2001). A comparative study conducted in Argentina, Brazil and Italy showed that human urinary sphinganine/sphingosine

ratios were significantly higher in populations consuming fumonisin contaminated maize compared to populations consuming hardly any maize (Solfrizzo et al., 2004). In a Croatian study on human serum and urinary sphingoid base levels from the Balkan endemic nephropathy area with presumed exposure to maize contaminated with fumonisins, it was concluded that impairment in the metabolism of sphingolipids might be considered as an early indicator of disease (Ribar et al., 2001).

Further studies are necessary to provide convincing evidence for using the sphinganine/ sphingosine ratio as a biomarker of human fumonisin exposure. The aim of this study was to compare the sphinganine and sphingosine levels as well as the sphinganine/sphingosine ratio in participants consuming subsistence grown fumonisin contaminated maize from a high (Centane) and low (Bizana) oesophageal cancer incidence area in the former Transkei region of the Eastern Cape Province of South Africa. Therefore, sphingosine and sphinganine levels and the sphinganine/sphingosine ratios were assessed in plasma and urine of male and female participants, while fumonisin levels were determined in maize samples collected contemporaneously from these regions.

Materials and methods

Chemicals

Sphinganine and sphingosine were obtained from Sigma Chemical Company (St. Louis, MO, USA). C20-sphinganine was a generous gift from Prof. A. H. Merrill Jr., (School of Biology, Parker H. Petit Institute for Bioengineering and Bioscience,

Georgia Institute of Technology, Atlanta, USA). All other chemicals and solvents were analytical grade from Merck (Darmstadt, Germany).

Sampling areas

Following ethical approval from the Medical Research Council and informed consent, 154 blood and 150 urine samples were collected from male and female participants during July 1997 from the Centane magisterial area, former Transkei region, Eastern Cape Province, South Africa. Home-grown maize was sampled from households in the same areas where these participants resided by random collection of visibly healthy maize (good maize) from 40 households. In addition, samples of visibly mouldy home-grown maize were hand selected from 31 of these households. In a similar manner 150 blood and 148 urine samples were collected from participants in the Bizana magisterial area of the former Transkei region during August 2000. As for the previous collection, 41 good and 30 mouldy home-grown maize samples were collected in Bizana and for comparison 41 good and 20 mouldy maize samples in Centane during July 2000.

Analytical methods

(i) Determination of sphinganine and sphingosine levels in plasma and urine

Sphinganine and sphingosine levels in plasma and urine were determined according to the method of Shephard and Van der Westhuizen (1998) with minor modifications. The concentrations were assessed by high-performance liquid chromatography with C20-sphinganine as an internal standard. The derivatised extracts were separated on a reversed-phase Synergi 4 μ Max-RP (75 x 4.6 mm I.D.) column (Phenomenex,

Torrance, CA, USA) and the isocratic mobile phase methanol/0.005 M potassium phosphate buffer (pH 3.35) (90:10) was pumped at a flow rate of 1 ml/min.

(ii) Determination of fumonisins in maize

Fumonisin levels in maize were determined according to the method of Shephard (2001). The derivatised extracts were separated on a reversed-phase Ultracarb 5 ODS (20) (150 x 4.6 mm I.D.) column (Phenomenex, Torrance, CA, USA) and the isocratic mobile phase of methanol/0.1 M sodium dihydrophosphate (pH 3.35) (77:23) was pumped at a flow rate of 1 ml/min.

(iii) Chromatography

The chromatographic system consisted of a Rheodyne 7725i injector (Cotati, CA, USA) with 200 µL loop, Waters Model 510 solvent delivery system (Milford, MA, USA), Borwin Chromatography Integration Software (Varian JBMS Developpements, Le Fontanil, France) and Waters Fluorescence 474 detector (excitation–335 nm and emission–440 nm).

Statistical analysis

The plasma and urine data were analysed within a multivariate General Linear Model (GLM), whereas the fumonisin data were compared using univariate GLM. The data were tested for normality and equality of variances, with natural log transformation of all variables, except that of the sphinganine/sphingosine ratio variable. Hochberg's GT2 method was used to analyse multiple comparisons, due to unequal cell sizes. The SPSS version 13 software package (SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

Results

The means and standard deviation of the sphinganine and sphingosine levels and the sphinganine/sphingosine ratios of the male and female participants of the two magisterial areas in plasma are shown in Table 1 and in urine in Table 2. Both the mean female ($p=0.01$) and male ($p>0.05$) plasma sphinganine levels were higher in Centane than in Bizana. The statistical insignificance of the difference in the male participants might be due to the large variation observed both in Centane (2.4–174 nM) and in Bizana (1.6–41 nM). Although the mean male and female sphingosine levels were higher in Centane than Bizana, this difference ($p>0.05$). was not significant. Significantly higher mean plasma sphinganine/sphingosine ratios in male as well as in female participants were detected in Centane compared to Bizana.

Chapter 5.1 - Table 1 Plasma (Sa) and sphingosine (So) levels and the Sa/So ratios from two magisterial areas in the former Transkei region of the Eastern Cape Province, South Africa*

Region	Gender	n	Sphinganine (nM)	Sphingosine (nM)	Sa/So Ratio
Centane 1997	Male	53	21.5±32.4aA ^{†‡}	80.1±93.1aA	0.34±0.24aA
	Female	99	16.5±19.4aA	72.2±78.6aA	0.30±0.27aA
	Combined	152	18.2±24.8A	75.0±83.7A	0.32±0.26A
Bizana 2000	Male	30	11.1±18.9aA	54.9±26.9aA	0.20±0.11aB
	Female	120	9.92±10.8aB	55.6±36.9aA	0.17±0.16aB
	Combined	150	10.16±10.5B	55.5±35.0A	0.18±0.15B

*Mean values ± standard deviations

[†]Lower case letters within columns: Means are significantly ($p<0.05$) different when followed by different letters for intra-magisterial area comparisons between males and females.

[‡]Upper case letters within columns: Means are significantly ($p<0.05$) different when followed by different letters for inter-magisterial area comparisons of males, females and combined groups.

However, within the magisterial areas the plasma sphingoid base parameters between male and female participants were very similar. As the female participants were about twice the number of male participants in Centane and four-fold the number of male participants in Bizana the combined male and female mean plasma sphinganine level was significantly higher in Centane than in Bizana. The mean combined plasma sphinganine/sphingosine ratio was also significantly higher in Centane than in Bizana.

Chapter 5.1 - Table 2 Urinary sphinganine (Sa) and sphingosine (So) levels and the Sa/So ratios from two magisterial areas in the former Transkei region of the Eastern Cape Province, South Africa*

Region	Gender	n	Sphinganine (nM)	Sphingosine (nM)	Sa/So Ratio
Centane 1997	Male	54	2.87±4.90aA†‡	14.2±22.3aA	0.31±0.34aA
	Female	93	5.61±7.79bA	26.4±32.6bA	0.27±0.20aA
	Combined	147	4.60±6.98A	21.9±29.8A	0.28±0.26A
Bizana 2000	Male	31	1.11±0.44aA	4.14±1.77aB	0.29±0.09aA
	Female	117	5.62±6.58bA	17.6±21.8bB	0.36±0.10aB
	Combined	148	4.68±6.13A	14.8±20.1B	0.34±0.10B

*Mean values ± standard deviations

†Lower case letters within columns: Means are significantly ($p < 0.05$) different when followed by different letters for intra-magisterial area comparisons between males and females.

‡Upper case letters within columns: Means are significantly ($p < 0.05$) different when followed by different letters for inter-magisterial area comparisons of males, females and combined groups.

A different result profile was observed in the urinary sphingoid base parameters compared to the plasma result profile (Tables 1 and 2). The mean male urinary sphinganine level was higher in Centane than in Bizana, but due to the large variation in Centane the difference was not significant. The female sphinganine

levels were almost identical in the two areas. Mean male as well as female urinary sphingosine levels and therefore the combined levels were significantly higher ($p<0.0001$) in Centane than in Bizana. This resulted in the mean male urinary sphinganine/sphingosine ratios being similar in Centane and Bizana; whereas the mean female ratios were significantly lower ($p<0.05$) in Centane than in Bizana.

Chapter 5.1 - Table 3 Fumonisin levels (mg/kg) in maize collected from two magisterial areas in the former Transkei region of the Eastern Cape Province, South Africa*

Region/ Maize	n	FB ₁ (mg/kg)	FB ₂ (mg/kg)	FB ₃ (mg/kg)	Total [†] Fumonisin (mg/kg)	Range of Fumonisin
Centane 1997						
Good	40	0.40±0.82	0.14±0.40	0.04±0.09	0.58±1.30	nd [†] -7.19
Mouldy	31	2.82±4.28	1.66±2.51	0.37±0.52	4.85±7.26	0.03-37.8
Centane 2000						
Good	41	0.55±1.06	0.28±0.70	0.054±0.12	0.88±1.78	nd-7.96
Mouldy	20	8.06±9.09	3.91±4.44	0.93±1.17	12.9±14.6	0.32-51.8
Bizana 2000						
Good	41	0.63±1.09	0.24±0.51	0.055±0.12	0.92±1.70	nd-6.41
Mouldy	30	5.75±7.41	2.88±3.75	0.74±1.04	9.37±12.1	0.39-53.0

*Mean values ± standard deviations are not significantly different for inter- or intra-magisterial area comparisons of good and mouldy maize.

[†]Total fumonisin represents the mean of all samples, with not detected taken as zero.

[†]nd = not detected (< 0.005 mg/kg)

Due to the higher number of female than male participants, the combined mean urinary sphinganine/sphingosine ratios were also significantly lower ($p<0.05$) in Centane than in Bizana. Furthermore, the mean urinary sphingosine levels in the male participants were significantly lower than the female participants within both the magisterial areas.

The percentage of good home-grown maize samples positive for fumonisins in both magisterial areas were very similar (38/40 and 37/41 in Centane in 1997 and 2000, respectively, and 40/41 in Bizana in 2000). The mean total fumonisin ($FB_1 + FB_2 + FB_3$) levels in the good maize collected from households in 1997 from Centane was lower than the mean fumonisin levels in the maize collected in 2000 from both the Bizana and Centane areas, whereas the mean total fumonisin levels in the mouldy maize in Centane in 2000 was more than two-fold higher than in 1997 (Table 3). However, no statistically significant differences were observed between the mean fumonisin levels in good home-grown maize or in mouldy home-grown maize between any of these collections.

Discussion

In this cross sectional study we observed that the plasma sphinganine and sphingosine levels of both the male and female participants were higher in the high oesophageal cancer incidence area of Centane than in the low incidence area of Bizana. However, these increased mean sphinganine levels were significantly higher only in the female participants, which were about twice the number of male participants in Centane and four-fold the number of male participants in Bizana. The relative number of male and female participants in this study, and other similar studies (Shephard et al., 2007; Van der Westhuizen et al., 1999), reflect the socio-economic situation in these regions where males are generally absent as migrant workers. Nevertheless, the plasma sphinganine/ sphingosine ratios in both the males and females, as well as the combined ratios, were significantly higher in Centane

than in Bizana. Previous studies conducted in these areas have shown that the home-grown maize consumed as the staple cereal in Centane was contaminated with higher levels of fumonisins than the maize consumed in Bizana (Marasas, 2001; Rheeder et al., 1992; Shephard et al., 2007).

Although the male urinary sphingosine and sphinganine levels were higher in Centane compared to Bizana, the difference in sphinganine, due to a large variation, was not significant. If the unanticipated higher mean female sphinganine/sphingosine ratio in Bizana were as a result of an increased mean sphinganine level in Bizana, it would have conformed to the expectation that the sphinganine/sphingosine ratios would be higher in an area where higher levels of fumonisin contaminated maize are consumed by the participants (Qui and Li, 2001; Riley et al., 1993; Solfrizzo et al., 2004). However, the higher female ratio was due to a significantly lower sphingosine level in Bizana compared to Centane, similarly to the mean male urinary sphingosine. Thus the plasma and urinary sphingosine levels in both males and females were higher in Centane than in Bizana. Serum sphingosine levels were correlated with age, tocopherols, carotenoids, selenium, retinol and cholesterol serum levels in a cross-sectional study conducted in a high oesophageal cancer risk area in Henan Province, China, whereas no associations were observed between serum sphinganine levels and demographic characteristics, diet and physiologic parameters (Abnet et al., 2001b). In this study, as observed in previous studies, the male participants had significantly lower urinary sphingosine levels than the female levels in both Centane and Bizana (Castegnaro et al., 1996; 1998; Van der Westhuizen et al., 1999).

The mean total fumonisin ($FB_1 + FB_2 + FB_3$) level in good home-grown maize collected from Centane in 1997 and 2000 was 580 and 880 $\mu\text{g/kg}$, respectively, whereas Bizana in 2000 had a mean total fumonisin level of 920 $\mu\text{g/kg}$. Previous studies conducted in Centane reported mean total fumonisin levels of 2110, 1975 and 1955 $\mu\text{g/kg}$ and 85, 565 and 930 $\mu\text{g/kg}$ in Bizana (Marasas, 2001; Rheeder et al., 1992; Rheeder et al. unpublished data). The mean total fumonisin levels for Centane in 1997 compared well with previously reported levels, whereas the level for Bizana in 2000 was higher than previously reported. Based on the mean total fumonisin level in good home-grown maize collected from Centane in 1997, 2000 and Bizana in 2000 and the assumption that a 60 kg person consumes 456 and 379 g of dry maize per day in Centane and Bizana, respectively (Shephard et al., 2007), the estimated probable daily intake (PDI) of fumonisins in these areas was 4.4, 6.7 and 5.8 $\mu\text{g/kg}$ body weight/day, respectively. The PDI for fumonisin in Centane in 1997 was marginally lower than that in Bizana. Therefore, the significantly higher combined mean male and female plasma sphinganine level of 18 nM, resulting in a mean combined plasma sphinganine/ sphingosine ratio of 0.32, in Centane than the sphinganine level of 10 nM, resulting in a 0.18 ratio, in Bizana, could not be ascribed to a higher fumonisin exposure level. However, the trend of higher PDI in Centane than in Bizana was again observed in 2000. In an extreme situation of low socio-economical status mouldy maize might be consumed, and then based on the above assumptions for this study, fumonisin exposure could result in estimated PDI of 36.9, 98.0 and 59.2 $\mu\text{g/kg}$ body weight/day, respectively. A recent study conducted in these areas observed mean PDI of 2.2 – 17.4 and 0.84 – 8.0 $\mu\text{g/kg}$ body weight/day in Centane and Bizana, respectively (Shephard et al., 2007). The mean estimated fumonisin exposure levels amongst consumers of beer in these areas could be 6.5

µg/kg body weight/day (Shephard et al., 2005). Consumption of beer brewed from mouldy home-grown maize will increase the PDI of fumonisins significantly. These studies show that in Centane and Bizana most of the fumonisin exposure levels were above the provisional maximum tolerable daily intake set by the Joint FAO/WHO Expert Committee on Food Additives of 2 µg total fumonisin/kg body weight/day (Bolger et al., 2001).

The combined urinary sphinganine/sphingosine ratios in Centane ranged from 0.02 to 1.70 compared to 0.12 to 0.65 in Bizana. These urinary sphinganine/sphingosine ratio ranges were higher than previously reported in China where the urinary ratio ranged from 0.028 to 0.25 prior to fumonisin exposure and following exposure of 0.4 to 740 µg FB₁/kg body weight/day, the urinary ratio ranged from 0.037 to 0.32, with a single exception which had a value of 0.87 (Qiu and Liu 2001). Given the very high levels of fumonisin exposure, these results would suggest that urinary sphinganine/sphingosine ratios would not be a viable and sensitive biomarker (Shephard et al., 2007). Participants from a study conducted in southern Brazil and northern Argentina exposed to mean fumonisin intakes of 0.57 and 0.55 µg FB₁/kg body weight/day, respectively, had mean urinary ratios of 1.57 and 0.69, respectively. In comparison, non-maize consuming participants from this study in southern Italy and central Argentina had a mean urinary ratio of 0.36, which was not significantly different from the ratio in participants from northern Argentina (Solfrizzo et al., 2004). It was thus concluded that the high sphinganine/sphingosine ratios observed in southern Brazil were not the result of fumonisin exposure, but of other unknown confounding factors.

Fumonisin exposure not only disrupts the base sphingolipids, but also results in decreased levels of the more complex sphingolipids causing a cascade of cellular effects, e.g. disruption of cellular membranes and effecting the homeostasis of cellular growth and differentiation (Riley et al., 2001; Merrill et al., 2001). However, the sampling from the two magisterial areas was from two different years and the fumonisin levels in maize fluctuate annually, thus complicating the investigation into the long term effect of fumonisin exposure and the consequence on the sphingoid bases and their role as a biomarker in humans. Therefore future studies should be aimed at a comparison of sphingolipid biomarkers and fumonisin exposure on an individual basis over an extended period.

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5.2

Individual fumonisin exposure and sphingoid base levels in rural populations consuming maize in South Africa

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Abstract

Low and high oesophageal cancer incidence areas of the former Transkei region of South Africa have been associated with corresponding low and high levels of fumonisin contaminated home-grown maize. This is the first study in South Africa assessing fumonisin B (FB) mycotoxin exposure by quantifying individual maize consumption with weighed food records and FB levels from maize in each participant's household and concurrently evaluating sphinganine sphingosine and sphinganine/sphingosine ratios in plasma and urine of these participants as possible biomarkers of FB exposure. The high consumption of maize in Bizana (n = 36) and Centane (n = 30) of 0.41 ± 0.21 and 0.39 ± 0.19 kg/day, respectively, confirms the reliance on maize as the dietary staple. Mean total FB (FB₁+FB₂+ FB₃) levels in home-grown maize were 0.495 ± 0.880 and 0.665 ± 0.660 mg/kg in Bizana and Centane, respectively. Mean fumonisin exposure based on individual consumption was 3.9 ± 7.3 and 4.1 ± 7.6 µg/kg body weight/day, respectively, for Bizana and Centane. The mean combined sphinganine/sphingosine ratios in Bizana and Centane were similar and ranged from 0.10-0.55 in plasma (n = 41) and urine (n = 62). There was no association between sphingoid base levels and/or sphinganine/sphingosine ratios in the plasma and urine and individual fumonisin exposure, negating the sphingoid bases as potential biomarkers of fumonisin exposure in humans.

Introduction

Fumonisin, mycotoxins mainly produced by *Fusarium verticillioides* and *F. proliferatum*, occur world-wide in maize and maize-based products intended for human consumption (Marasas, 2001; Shephard et al., 1996a). Fumonisin B₁, B₂ and B₃ (FB₁, FB₂ and FB₃) are the major naturally occurring fumonisin analogues in maize. Oesophageal and liver cancer as well as neural tube defects in humans have been linked to fumonisins (Marasas et al., 2004; Rheeder et al., 1992; Sun et al., 2007). The former Transkei region, Eastern Cape, South Africa, has one of the highest oesophageal cancer incidence rates in the world (Makaula et al., 1996). Early studies in the region reported a 20-fold lower oesophageal cancer incidence rate in the Bizana than in the Centane magisterial district. Even though more recent studies have shown a rising incidence rate in Bizana, Centane has maintained consistently higher oesophageal cancer incidence rates (Makaula et al., 1996; Somdyala et al., 2003a,b).

Previous studies have shown that fumonisin levels in home-grown maize were up to 25-fold lower in Bizana than in Centane (Rheeder et al., 1992), whereas a more recent study has shown similar high fumonisin levels in both areas (Van der Westhuizen et al., 2008). High levels of fumonisins have also been reported in naturally contaminated maize from other areas where high incidences of oesophageal cancer occur, viz., Charleston, SC, USA; Cixian County, Fusui County, Guangxi Autonomous Region and Huaian County, Jiangsu Province, China; Northern Italy and Santa Catarina, Southern Brazil (Doko and Visconti, 1994; Sun et al., 2007; Sydenham et al., 1991; Van der Westhuizen et al., 2003). The International

Agency for Research on Cancer (IARC) declared FB₁ as a group 2B carcinogen (possibly carcinogenic in humans) (IARC, 2002). The 56th meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) determined a group provisional maximum tolerable daily intake (PMTDI) for FB₁, FB₂ and FB₃, alone or in combination, of 2 µg/kg body weight/day (Bolger et al., 2001).

Fumonisin inhibit the key enzyme, ceramide synthase, in the de novo sphingolipid pathway leading to a disruption in levels of the sphingoid bases in mammalian cells. The ratios of the sphingoid bases, sphinganine and sphingosine, in plasma and urine of various animal species have been reported as possible biomarkers of PDI (Riley et al., 1994; Shephard et al., 1996b; Van der Westhuizen et al., 2001; Wang et al., 1992). However, subsequent studies conducted in various human populations exposed to different levels of fumonisin have not been able to show that sphinganine or sphingosine levels or the sphinganine/sphingosine ratio can be utilised as a biomarker for fumonisin exposure (Qiu and Liu, 2001; Solfrizzo et al., 2004; Silva et al., 2009; Van der Westhuizen et al., 1999, 2008). Previous studies comparing the Bizana and Centane populations as a whole failed to find an association between calculated fumonisin exposure (based on mean fumonisin levels of maize collected in the respective areas and an estimated mean maize consumption) and the mean plasma or urinary sphinganine/sphingosine ratios (Van der Westhuizen et al., 1999, 2008).

The aim of this study was to assess the potential of plasma and urinary sphinganine and sphingosine levels and/or sphinganine/sphingosine ratios as biomarkers of fumonisin exposure at an individual level within the Bizana and Centane populations.

Fumonisin exposure was determined individually from maize consumption and fumonisin levels in maize from the participants' household.

Materials and Methods

Chemicals

Fumonisin (FB₁, FB₂ and FB₃) standards were isolated (>95% purity) at the PROMEC Unit according to the method of Cawood et al. (1991). Sphinganine and sphingosine were obtained from Sigma Chemical Company (St. Louis, MO, USA) and C20-sphinganine standard (D-erythro-C20-dihydro-sphingosine) from Matreya Inc. (Pleasant Gap, PA, USA). All other chemicals and solvents were analytical grade from Merck (Darmstadt, Germany).

Study population and sampling

The study was approved by the Ethics Committee of the Medical Research Council and informed consent was obtained from each participant. Participants were drawn over the study period (2001–2003) from selected households in north-western Bizana (n = 24) and south-eastern Centane (n = 24) magisterial districts, former Transkei Region, Eastern Cape Province, South Africa. The total number of participants during the study period were 8 male and 28 female participants from Bizana of whom blood (n = 21, 2001–2002) and spot urine (n = 36, 2001–2003) samples were collected randomly during the day and 11 male and 20 female participants from Centane of whom blood (n = 20) and urine (n = 26) samples were collected similarly. The maize sampled from the participants' households during the

study period was healthy home-grown maize used to prepare maize-based meals at the time of collection. Where home-grown maize was not available, commercial maize (meal or samp, dehulled dried maize kernels that have been stamped broken into large pieces) were collected from the households. In household with both home-grown and commercial maize both kinds were collected (see Table 3).

Analytical methods

(i) Determination of fumonisins in maize

Fumonisin levels in maize were determined according to the method of Shephard (2001). Briefly, maize was ground in a laboratory mill to a fine meal and extracted with 100 mL methanol/water by homogenization. A 10 mL aliquot was applied to a strong anion exchange solid phase extraction cartridge and the fumonisins were eluted with acetic acid in methanol. The purified extract was evaporated to dryness with nitrogen gas at 60 °C and the dried residue was stored at 4 °C awaiting chromatographic analysis. The redissolved (200 µL methanol) extracts were derivatised and separated (20 µL injection) on a reversed-phase Luna 4µ C18 (2) (150 x 4.6 mm I.D.) column (Phenomenex, Torrance, CA, USA) and the isocratic mobile phase of methanol/0.1 M sodium dihydrogenphosphate (pH 3.35) (77:23) was pumped at a flow rate of 1 mL/min. The chromatographic system consisted of a Rheodyne 7725i injector (Cotati, CA, USA), Waters Model 510 solvent delivery system (Milford, MA, USA), Borwin Chromatography Integration Software (Varian JBMS Developpements, Le Fontanil, France) and Waters Fluorescence 474 detector (excitation – 335 nm and emission – 440 nm).

(ii) Determination of sphinganine and sphingosine levels in plasma and urine

Sphingoid bases were determined according to the method of Castegnaro et al. (1998) with minor adjustments. Briefly, sphinganine and So were extracted from plasma and urine samples by shaking with ethyl acetate. The organic layer was separated by centrifugation (500 g, 10 min, 4 °C) and evaporated to dryness with nitrogen gas below 40 °C. The dried residue was redissolved in a potassium hydroxide/chloroform solution and hydrolysed at 37 °C for 90 min. After incubation the hydrolysed residue was washed with alkaline water and centrifuged (500 g, 10 min, 4 °C). The chloroform layer was evaporated to dryness with nitrogen gas at 40 °C and stored at 4 °C awaiting chromatographic analysis. The redissolved (275 µL methanol) extracts were derivatised with o-phthaldialdehyde and separated (50–75 µL injection) on the same HPLC system used for the fumonisin analyses with a reversed-phase Synergi 4 µ Max-RP (75 x 4.6 mm I.D.) column (Phenomenex, Torrance, CA, USA) and an isocratic mobile phase of methanol/5 mM potassium phosphate buffer (pH 3.35) (90:10) pumped at a flow rate of 1 mL/min. Quantification was performed with C20– sphinganine as an internal standard.

Fumonisin exposure

Individual fumonisin exposure for each participant was calculated as µg/kg body weight/day based on the total fumonisin ($TFB = FB_1 + FB_2 + FB_3$) level in healthy home-grown and/or commercial maize collected from each participant's household. At the time of collection most of the participants were consuming food prepared from home-grown maize as opposed to commercial maize. Maize consumption was assessed by acquiring weighed food records for each member of the household with respect to maize dishes (Shephard et al., 2007).

Statistical analysis

The data were analysed within a one-way Analysis of Variance (ANOVA) model. The data was tested for normality and equality of variances, with natural log transformation of all variables. The Stata/IC version 10.1 software package (StataCorp, Texas, USA) was used for statistical analysis.

Results

Study population, maize intake and fumonisin exposure

The participants were predominantly female, particularly from Bizana, primarily due to migrant labour and therefore participating males tended to be older. The combined mean age of the Bizana participants was 49 ± 17 years compared to the 48 ± 18 years of Centane (Table 1).

Chapter 5.2 - Table 1 The combined (2001–2003) age and weight (mean \pm standard deviation) in male and female participants from the two magisterial districts in the former Transkei.

Region	Gender	n	Age (years)	Weight (kg)
Bizana	Male	8	61 ± 14	63 ± 10
	Female	28	46 ± 17	75 ± 23
	Combined	36	49 ± 17	72 ± 21
Centane	Male	11	48 ± 19	66 ± 14
	Female	20	48 ± 18	69 ± 10
	Combined	31	48 ± 18	68 ± 12

Means are not significantly different.

In Bizana the combined mean body weights was 72 ± 21 kg, whereas in Centane it was 68 ± 12 kg. The mean maize intakes for males (0.36 ± 0.22) and females (0.43 ± 0.21 kg/day) in Bizana were similar to that obtained in Centane which were 0.44 ± 0.19 and 0.37 ± 0.19 kg/day, respectively, for males and females (Table 2).

Chapter 5.2 - Table 2 The combined (2001–2003) maize intake and probable daily intake (PDI) (mean \pm standard deviation) in male and female participants from the two magisterial districts in the former Transkei

Region	Gender	n	Maize intake (kg/day)	n	PDI ¹ (μ g/kg bw/day)	PDI ¹ Range (μ g/kg bw/day)	Participants exceeding PMTDI ² (n)
Bizana							
	Male	8	0.36 ± 0.22	7	4.8 ± 7.5	0.49–21	2
	Female	28	0.43 ± 0.21	23	3.6 ± 7.4	> 0.01–29	6
	Combined³	36	0.41 ± 0.21	30	3.9 ± 7.3	> 0.01–29	8
Centane							
	Male	10	0.44 ± 0.19	10	1.9 ± 2.0	0.04–5.5	4
	Female	20	0.37 ± 0.19	20	5.2 ± 9.1	0.21–35	8
	Combined	30	0.39 ± 0.19	30	4.1 ± 7.6	0.04–35	12

¹PDI based on mean total fumonisin levels, maize intake and individual body weight (bw) (μ g/kg body weight/day)

²Provisional maximum tolerable daily intake (PMTDI) = 2 μ g fumonisin/kg bw/day (JECFA)

³Combined = Combining the results obtained for the male and female participants
Means are not significantly ($p > 0.05$) different within nor between magisterial districts

The maize samples collected in the two magisterial districts over the three harvesting seasons (2001–2003) had a wide range of fumonisin levels, due to seasonal

variation, resulting in large standard deviations (Table 3). The mean TFB level of the home-grown maize in the 2003 season compared with the 2001 and 2002 seasons were significant lower ($p < 0.05$) in Bizana and although the 2002 and 2003 seasons also had lower levels than 2001 in Centane, these differences were not significantly ($p > 0.05$) different. In 2003 the mean TFB of the home-grown maize was marginally ($p = 0.57$) higher in Centane than in Bizana. There was no significant difference ($p =$

Chapter 5.2 - Table 3 The total fumonisin levels (mean \pm standard deviation) in home-grown (separately) and commercial (combined) maize from the two magisterial districts in the former Transkei.

Region	Maize Season	n	Total fumonisin ¹ (mg/kg)	Range (mg/kg)
Bizana	Home-grown			
	2001	7	1.020 \pm 1.465a ²	0.015–3.975
	2002	9	0.485 \pm 0.525a	0.045–1.430
	2003	9	0.090 \pm 0.160A ³ b	nd ⁴ –0.500
	Total	25	0.495 \pm 0.880	nd–3.975
Centane	Home-grown			
	2001	3	1.140 \pm 0.705a	0.720–1.955
	2002	9	0.540 \pm 0.675a	0.040–1.980
	2003	3	0.565 \pm 0.560aB	0.195–1.205
	Total	15	0.665 \pm 0.660	0.040–1.980
Bizana	Commercial			
	2001-2003	9	0.530 \pm 0.520	0.130–1.580
Centane	Commercial			
	2001-2003	11	0.235 \pm 0.305	0.005–0.900
Combined	Commercial	20	0.370 \pm 0.430	0.005–1.580

¹Mean total fumonisin (FB₁ + FB₂ + FB₃)

²Lower case letters: Means in a column are significantly ($p < 0.05$) different when followed by different letters for seasonal comparison in Centane

³Upper case letters: Means in a column are marginally ($p = 0.57$) different when followed by different letters for seasonal comparison between areas.

⁴nd = not detected (< 0.005 mg/kg)

0.062) in the mean (2001–2003) TFB levels of the home-grown maize between Bizana and Centane. The mean TFB in commercial maize (2001–2003) from Bizana compared to Centane were also not significantly different ($p > 0.05$). Although there were also no significant difference between the mean TFB (2001–2003) of the home-grown maize and the commercial maize within the areas, the TFB in Bizana commercial maize ($n = 9$) was higher ($p > 0.05$) than in home-grown maize ($n = 25$), whereas in Centane the TFB in commercial maize ($n = 11$) was lower ($p > 0.05$) than in home-grown maize ($n = 15$). The most abundant fumonisin analogue, FB₁, contributed 76% of the TFB contamination with 58/60 maize samples positive for fumonisin (results not shown). The mean probable daily intake (PDI) of fumonisin by the combined participants in Bizana and Centane were also similar at 3.9 ± 7.3 and 4.1 ± 7.6 $\mu\text{g/kg}$ body weight (bw)/day, respectively, with a range of < 0.1 – 35 $\mu\text{g/kg}$ bw/day for the entire study period (Table 2).

Sphingoid base levels and ratios in plasma and urine

There were no significant differences in gender or season (data not shown) in the plasma sphingoid bases, sphinganine and sphingosine, or in the sphinganine/sphingosine ratio within or between areas (Table 4). The sphinganine levels for all participants ranged from 1.9 to 10 and 1.1–21 nM in Bizana and Centane, respectively, compared to the sphingosine ranging from 8.3 to 40 and 4.8 to 52 nM, respectively, (ranges not shown in Table 4). The sphinganine/sphingosine ratios were similar within and between the magisterial districts ranging from 0.10 to 0.51 standard deviations. When comparing the plasma to urinary sphingoid bases the levels were also similar, except for the ten- and fivefold lower sphinganine and sphingosine level, respectively, in the Centane males.

Chapter 5.2 - Table 4 The combined plasma (2001-2002) and urinary (2001-2003) sphinganine and sphingosine levels and the sphinganine/sphingosine (Sa/So) ratios (mean \pm standard deviation) in male and female participants from the two magisterial districts in the former Transkei.

Plasma/ Urine	Region/ Gender	n	Sphinganine (nM)	Sphingosine (nM)	So/Sa Ratio
Plasma	Bizana				
	Male	7	4.4 \pm 1.4	19 \pm 10	0.26 \pm 0.10
	Female	14	6.3 \pm 2.8	22 \pm 10	0.30 \pm 0.08
	Combined ¹	21	5.6 \pm 2.5	21 \pm 10	0.29 \pm 0.09
Plasma	Centane				
	Male	8	7.2 \pm 6.4	23 \pm 16	0.29 \pm 0.10
	Female	12	6.9 \pm 6.5	21 \pm 12	0.27 \pm 0.12
	Combined	20	7.0 \pm 6.3	22 \pm 14	0.28 \pm 0.11
Urine	Bizana				
	Male	8	3.4 \pm 7.0	21 \pm 46	0.17 \pm 0.09
	Female	28	8.1 \pm 16	34 \pm 68	0.22 \pm 0.08
	Combined	36	7.1 \pm 14	31 \pm 63	0.21 \pm 0.08
Urine	Centane				
	Male	8	0.72 \pm 0.55a ²	4.1 \pm 1.9a	0.17 \pm 0.10
	Female	18	4.6 \pm 5.1b	20 \pm 22b	0.21 \pm 0.13
	Combined	26	3.4 \pm 4.6	15 \pm 20	0.20 \pm 0.12

¹Combined = Combining the results obtained for the male and female participants

²Means in a column are significantly ($p < 0.05$) different when followed by different letters

A different urinary sphingoid base profile to that in plasma was observed when comparing male and female levels. Although the sphinganine and sphingosine levels were lower in males than females in Bizana ($p > 0.05$) and Centane ($p < 0.05$); the sphinganine/sphingosine ratios were similar between the areas. The higher urinary sphinganine and sphingosine levels in both males and females in Bizana compared to Centane were not significant, due to the large standard deviations. When

comparing the plasma to urinary sphingoid bases the levels were also similar, except for the ten- and fivefold lower Sa and So level, respectively, in the Centane males.

Chapter 5.2 - Table 5 Comparison of the respective plasma and urinary sphinganine/ sphingosine (Sa/So) ratios (mean \pm standard deviation) for probable daily intakes (PDI) above and below the provisional maximum tolerable daily intake (PMTDI).

Region	n	PDI ¹ ($\mu\text{g/kg bw/day}$)	n	Plasma ratio (Sa/So)	n	Urine ratio (Sa/So)
PDI below PMTDI²						
Bizana	22	0.71 \pm 0.61	14	0.30 \pm 0.08	22	0.20 \pm 0.09
Centane	18	0.83 \pm 0.68	9	0.26 \pm 0.10	13	0.21 \pm 0.14
Combined³	40	0.77 \pm 0.64	23	0.28 \pm 0.09	35	0.20 \pm 0.11
PDI above PMTDI						
Bizana	8	12.6 \pm 10.1	7	0.26 \pm 0.09	8	0.21 \pm 0.09
Centane	12	9.1 \pm 10.3	11	0.30 \pm 0.12	12	0.19 \pm 0.12
Combined	20	10.5 \pm 10.1	18	0.29 \pm 0.11	20	0.20 \pm 0.11

¹Probable daily intake (PDI) based on mean total fumonisin levels, maize intake and individual body weight (bw) ($\mu\text{g/kg bw/day}$)

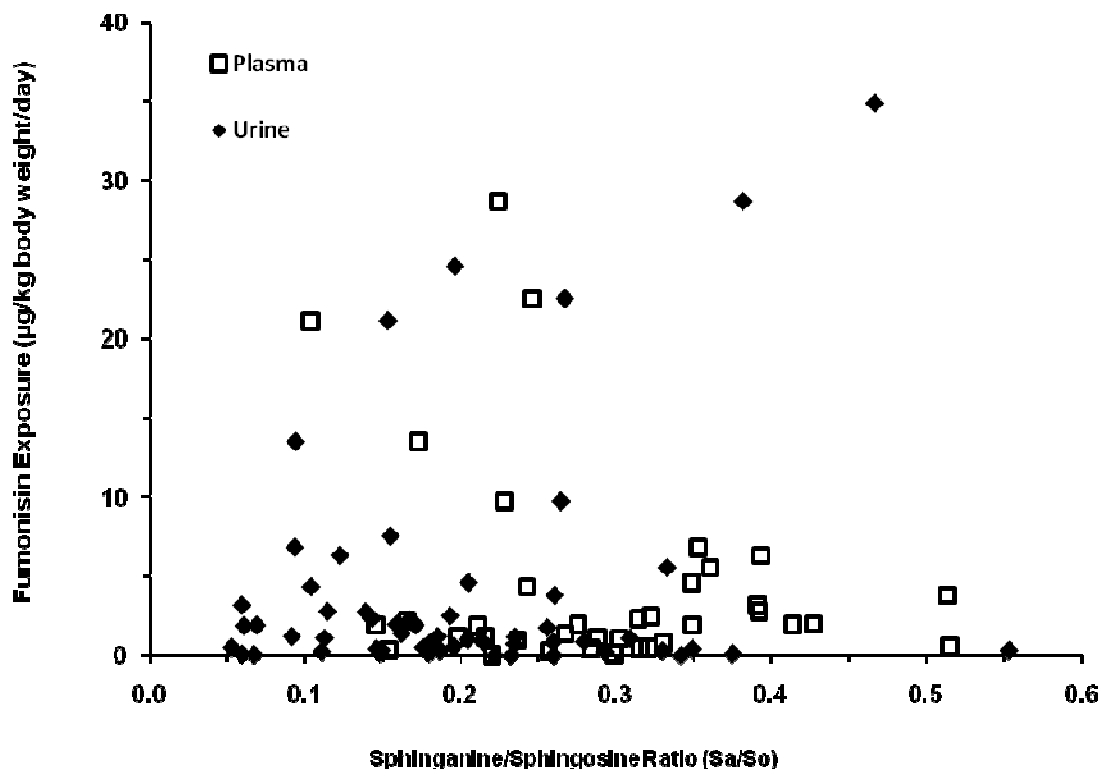
²Provisional maximum tolerable daily intake (PMTDI) = 2 μg fumonisin/kg bw/day (JECFA)

³Combined = Combining the results obtained for Bizana and Centane magisterial districts

Means are not significantly ($p > 0.05$) different within nor between magisterial districts

The participants were divided into groups with PDIs below and above the PMTDI determined by JECFA (Table 5). There was no difference between the mean plasma and urinary sphinganine/sphingosine ratios for PDIs above or below the PMTDI. Individual fumonisin exposures compared to the individual sphinganine/sphingosine ratios for all the participants from the Bizana and Centane magisterial districts in

plasma (2001–2002, $r = 0.1098$, $p > 0.05$,) and urine (2001–2003, $r = 0.0638$, $p > 0.05$) failed to show a relationship ($p > 0.05$) (Figure 1).



Chapter 5.2- Figure 1 Individual fumonisin exposures compared to the individual sphinganine/ sphingosine ratios for all the participants from the Bizana and Centane magisterial districts in plasma ($r = 0.1098$, $p > 0.05$) and urine ($r = 0.0638$, $p > 0.05$).

Discussion

This is the first study in Southern Africa assessing individual fumonisin exposure to evaluate sphinganine, sphingosine and sphinganine/sphingosine ratios in plasma and urine of each participant as possible biomarkers of FB exposure in humans. The

mean dry maize intake for all participants over the entire study period was 0.41 and 0.39 kg/day in Bizana and Centane, respectively. This is comparable to a previous study using the same food consumption methodology conducted on a larger population in these areas which reported mean intake levels of 0.38 and 0.46 kg/day, respectively (Shephard et al., 2007). The high consumption emphasizes that maize is almost the sole source of food in these rural areas and that the mean fumonisin levels in both Bizana and Centane would result in PDIs exceeding the JEFCA PMTDI. The standard set by the FDA for the United States is 2 mg TFB/kg maize and the European Commission regulation is 1 mg TFB/kg maize intended for direct human consumption (European Commission Regulations, 2007; FDA, 2001). Based on results obtained from this study in Centane, a 68 kg participant consuming 0.39 kg maize/day at 1 mg TFB/kg maize (European Commission regulation) would result in a PDI of 5.7 µg/kg body weight/day. This participant would have to consume less than 0.14 kg maize/day to achieve a PDI below the PMTDI at the regulatory level set by the European Commission. Even a participant from Bizana (72 kg) consuming commercial maize at the current TFB (0.495 mg/kg) would result in a PDI of 2.8 µg/kg body weight/day. Furthermore, regulating fumonisin levels in maize at a national level will have no effect on fumonisin exposure in rural communities consuming large quantities of maize (Gelderblom et al., 2008). Therefore finding measures to reduce fumonisin contamination of maize in this region is imperative.

Seasonal variation of the fumonisin levels in the maize from this area is evident from the current study which is in agreement with previous studies (Van der Westhuizen et al., 2008). Despite the large seasonal variations in fumonisin contamination of the home-grown maize within and over the three study seasons, the combined mean

fumonisin exposure was similar in Bizana and Centane. Bizana had a higher mean TFB level in 2001 than previously reported in this area and Centane had lower levels in 2002 and 2003 than ever reported (Rheeder et al., 1992; Marasas, 2001, Van der Westhuizen et al., 2008). Maize from Bizana in 2003 had a significantly lower TFB level than maize in Centane and was comparable to the lowest level reported for Bizana, conforming in 2003 to the previously observed pattern for this region (Rheeder et al., 1992). In the current study fumonisin exposure was assessed at 3.9 µg/kg bw/day in Bizana and 4.3 µg/kg bw/day in Centane. Comparative fumonisin exposure levels 3.4 (Bizana) and 8.7 (Centane) µg/kg bw/day, were obtained in an earlier study estimating PDI, assuming an adult body weight of 60 kg and a mean fumonisin level calculated from all previous studies conducted in these areas (Shephard et al., 2007).

The combined PDI in each area was approximately double the JECFA PMTDI; with 20/59 participants exposed to levels exceeding the PMTDI showing that certain individual participants were exposed to extremely high levels of fumonisin. As studies in experimental animals showed that fumonisin exposure causes an increase in sphingoid base levels in plasma and urine, specifically in sphinganine and thus the sphinganine/sphingosine ratio (Enongene et al., 2002; Van der Westhuizen et al., 2001; Wang et al., 1999), this study investigated the sphingoid levels and ratios as potential biomarkers of fumonisin exposure. It was hypothesised that the sphinganine levels and the sphinganine/sphingosine ratio in plasma and urine would be elevated in participants exposed to higher fumonisin levels. A previous study observed that there was no relationship between estimated mean fumonisin exposures (based on mean fumonisin levels and mean maize consumption) within

communities and the mean plasma and urinary sphingoid bases and sphinganine/sphingosine ratios in males and females (Van der Westhuizen et al., 2008). This study measured sphingoid bases and fumonisin exposure on an individual basis enabling direct comparison. However, despite a very wide range of fumonisin exposure, sphinganine, sphingosine and sphinganine/sphingosine ratios failed to show a relationship with FB exposure. In addition, neither plasma nor urinary sphingoid base profiles showed any association with the extent of exposure. Even comparison of the PDI of the participants exposed above and below the PMTDI did not show any difference in plasma or urinary sphingoid base profiles. Therefore, the sphingoid bases and ratios could not be associated with the fumonisin exposure in this study. This is in agreement with similar studies conducted in Burkino Faso; Henan, China and Southern Italy, Southern Brazil and Central Argentina (Nikiéma et al., 2008; Qiu and Liu, 2001; Silva et al., 2009; Solfrizzo et al., 2004).

Despite the large intra- and inter seasonal variation in fumonisin contamination of the home-grown maize as well as the large variation in individual sphingoid base levels, the combined fumonisin exposure levels and sphinganine/sphingosine ratios between the areas were similar. In conclusion, neither plasma nor urinary sphingoid base levels and sphinganine/sphingosine ratios could be associated with fumonisin exposure on an individual basis and therefore negated the sphingoid bases as potential biomarkers in humans. Future studies should investigate alternative biomarkers and a very recent study in Mexico has indicated that FB₁ in urine may be more promising to monitor fumonisin exposure in humans (Gong et al., 2008).

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Conflicts of interest

The authors report no financial or conflicts of interest.

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6

**Reducing fumonisin exposure in a
maize subsistence community**

6.1

Optimising sorting and washing of home-grown maize to reduce fumonisin contamination under laboratory-controlled conditions

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Abstract

Subsistence farming communities with low socio-economic status reliant on a mono cereal maize diet are exposed to fumonisin levels that exceed the provisional maximum tolerable daily intake of 2 µg/kg body weight/day recommended by the Joint FAO/WHO Expert Committee on Food Additives. In the rural Centane magisterial district, Eastern Cape Province, South Africa, it is customary during food preparation to sort visibly infected maize kernels from good maize kernels and to wash the good kernels prior to cooking. However, this customary practice seems not to sufficiently reduce the fumonisin levels. This is the first study to optimise the reduction of fumonisin mycotoxins in home-grown maize based on customary methods of a rural population under laboratory-controlled conditions. Maize obtained from subsistence farmers was analysed for the major naturally occurring fumonisins (FB₁, FB₂ and FB₃) by fluorescence HPLC. Large variations were observed in the unsorted and the experimental maize batches attributable to the non-homogeneous distribution of fumonisin contamination in maize kernels. Optimised hand-sorting of maize kernels by removing the visibly infected/damaged kernels (fumonisins, 53.7 ± 15.0 mg/kg, 2.5% by weight) reduced the mean fumonisins from 2.32 ± 1.16 mg/kg to 0.68 ± 0.42 mg/kg. Hand washing of the sorted good maize kernels for a period of 10 min at 25°C resulted in optimal reduction with no additional improvement for wash periods up to 15 h. The laboratory optimised sorting reduced the fumonisins by 71 ± 18% and an additional 13 ± 12% with the 10 min wash. Based on these results and on local practices and practicalities the protocol that would be recommended to subsistence farmers consists of the removal of the infected/damaged kernels from the maize followed by a 10 min ambient temperature water wash.

Introduction

Fumonisin, carcinogenic mycotoxins, occur worldwide in maize and maize-based products consumed by humans (Shephard et al., 1996). Fumonisin B₁, B₂ and B₃ (FB₁, FB₂ and FB₃) are the major naturally occurring fumonisin analogues in maize (Shephard et al., 1996). The most abundant of the fumonisin mycotoxins, FB₁, was evaluated by the International Agency for Research on Cancer as a Group 2B carcinogen, i.e. possibly carcinogenic to humans (IARC, 2002). Several studies have reported an association between high incidence rates of oesophageal cancer and consumption of fumonisin contaminated maize by rural populations, e.g., Eastern Cape Province, South Africa; Jiangsu Province, China and Santa Catarina, Brazil (Marasas, 2001; Sun et al., 2007; Van der Westhuizen et al., 2003). Fumonisin exposure has also been associated with primary liver cancer in Jiangsu Province, China (Sun et al., 2007), and has been implicated in the aetiology of neural tube defects (Marasas et al., 2004).

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has determined a group provisional maximum tolerable daily intake (PMTDI) for fumonisin B₁, B₂ and B₃, alone or in combination, of 2 µg/kg body weight/day (Bolger et al., 2001). As maize is a dietary staple in sub-Saharan Africa, chronic exposure to high levels of these potent mycotoxins is a regular occurrence (Shephard et al., 2006). Recent studies conducted in populations at high risk for oesophageal cancer from the Centane magisterial district of the Eastern Cape Province, South Africa, reported mean probable daily intake (PDI) levels of 4.4-8.7 µg/kg body weight/day (Shephard et al., 2007; Van der Westhuizen et al., 2008). The high level of fumonisin exposure

in these populations, reliant on subsistence maize as a major dietary staple, stresses the importance of reducing exposure and thereby lowering the associated risk for disease development. This is of particular importance as international regulations on fumonisin in maize will have no effect on exposure levels in these rural populations consuming large quantities of home-grown maize daily (Gelderblom et al., 2008; Marasas et al., 2008; Wild and Gong, 2010).

Reduction of mycotoxin exposure and related health effects in subsistence farming communities can realistically only be based on practical low-cost measures. An intervention study in Guinean villages resulted in a 60% aflatoxin reduction in groundnuts by introducing practical primary prevention measures based on improved storage (Turner et al., 2005). In contrast to aflatoxin, where unsuitable storage practices contribute to increased levels, fumonisin contamination is mainly produced prior to harvesting (Wild and Gong, 2010). Fandohan et al. (2005) reported that the processing procedures for traditionally prepared maize meal dishes from Benin reduced fumonisin levels in maize by up to 87% depending on the specific food type. Sorting, winnowing, washing and dehulling of maize kernels were very effective in achieving these reductions, but the actual cooking process did not achieve a reduction. In contrast a 23% fumonisin reduction due to the cooking process in traditionally prepared stiff porridge prepared from South African commercial maize meal was reported (Shephard et al. 2002).

Although the traditional maize food preparations in the communities of the Centane magisterial district includes the sorting of maize on the cob (ear) into visibly healthy (good) and visibly fungal infected (mouldy) maize this customary practice does not

seem to sufficiently reduce the fumonisin levels (Kimanya et al., 2008a; Shephard et al., 2005; Van der Westhuizen et al., 2008). The aim of this study was to assess the customary maize food preparation practices and subsequently to optimise the reduction of fumonisin contamination in home-grown maize by hand-sorting and washing of maize kernels under laboratory-controlled conditions. The ultimate goal was to be able to recommend a means of fumonisin reduction, which will be both culturally acceptable and practically implementable, in the subsistence farming communities.

Methods and Materials

Chemicals

Fumonisin (FB₁, FB₂ and FB₃) standards were isolated at the PROMEC Unit according to the method of Cawood et al. (1991). All other chemicals and solvents were analytical grade from Merck (Darmstadt, Germany).

Focus groups and maize collection

Focus groups (n = 17), consisting of 6-10 females each that traditionally prepared the maize meals were recruited from the Centane magisterial district, Eastern Cape Province, South Africa. The interviews were conducted in the local language (isiXhosa) by the trained fieldworker with a questionnaire on the customary sorting and washing of maize kernels prior to cooking maize-based meals. It is the norm in this area to sort the unshelled maize cobs into “fit for human consumption” (good maize) and “visibly infected” (mouldy maize) groups. Good home-grown maize, as

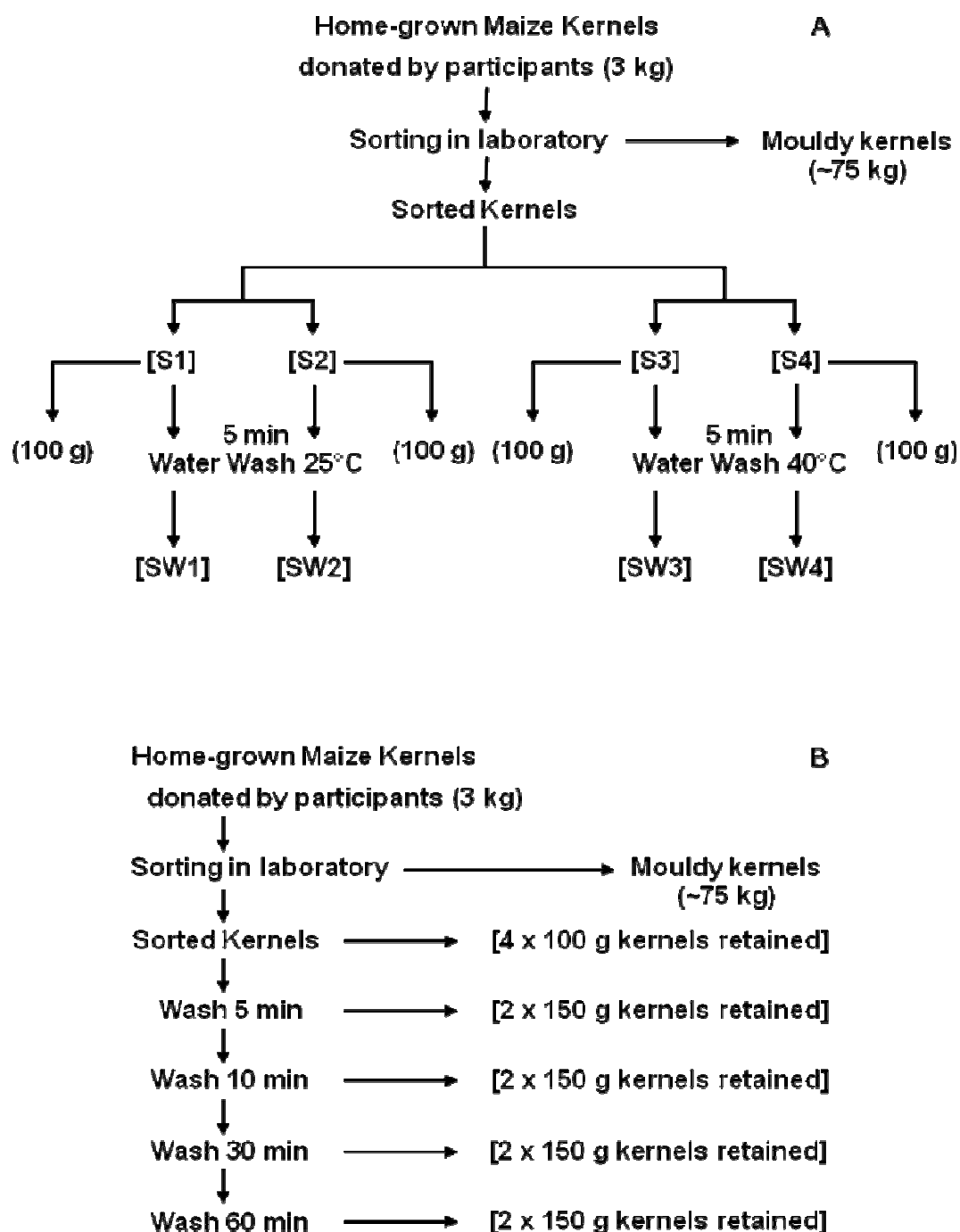
separated by the participants, was donated for the laboratory study and a commensurate amount of rice was provided. The home-grown maize cobs were shelled, the kernels pooled and stored at 4°C prior to laboratory experiments.

Mycological analyses

Home-grown maize was mycologically analyzed for fungal incidence (% kernels infected). Briefly, a random kernel subsample (80-100 g) was surface sterilised for 1 min in 3.5% commercial sodium hypochlorite solution and rinsed twice in sterile water. One hundred kernels (5 kernels/ 90 mm petri-dish) were transferred to malt extracted agar (1.5%) containing novobiocin (150 mg/L) prior to incubation at 25°C for 5 to 7 days. All the isolated fungi were recorded and their frequencies determined using a stereo-microscope. *Fusarium* species were identified according to Leslie and Summerell (2006) and other fungi were identified on the basis of their morphology and characteristics in culture, i.e., *Aspergillus* and *Stenocarpella* species.

Hand sorting of home-grown maize kernels

Following shelling of the donated good home-grown maize cobs, the kernels (approximately 23 kg) were thoroughly and repeatedly mixed, whereafter six maize subsamples (500 g) were randomly selected and stored at 4°C prior to fumonisin analyses. Four maize kernel batches (3 kg each) were also randomly selected from the pooled home-grown maize. The maize kernels from each batch were separately hand-sorted on the laboratory bench. Visibly infected/damaged kernels were removed by hand from each kernel batch and separately stored at 4°C for duplicate fumonisin analyses. The remaining sorted good kernels from each batch were



Chapter 6.1 - Figure 1 Flow diagram of the wash experiments in Table 3. The size of the subsamples is indicated in brackets at each fraction. (A) Flow diagram of the temperature experiment conducted in duplicate on each of two 3 kg batches. (B) Flow diagram of the hour experiment (The day experiment was conducted similarly).

utilised for the washing experiments. Fumonisin (FB₁ + FB₂ + FB₃) were expressed as mg/kg maize.

Washing Experiments

Effect of Temperature

The effect of water temperature on fumonisin reduction was investigated at 25°C and 40°C. The use of ambient (25°C) water was selected as it precludes the necessity of heating water and the higher temperature was selected as it would not be too warm for hand washing. Two of the sorted good kernel batches were subdivided into four subsamples (~ 750 g each, Figure 1A). Aliquots (100 g kernels) of each of the subsamples were stored at 4°C prior to fumonisin analyses. Two subsamples from each batch were washed at each selected temperature using water to maize ratios of 1:1. The washing procedure consisted of a 5 min hand agitation. The washed maize kernels were dried on paper towels on the laboratory bench at room temperature overnight (> 18 h) and stored at 4°C prior to fumonisin analyses.

Effect of Wash Duration

The effect of the duration of the water washes on fumonisin reduction was investigated at 5, 10, 30 and 60 min. Four randomly selected subsamples (100 g kernels) of the third good kernel sample batch were stored at 4°C for fumonisin analyses (S1-S4, Figure 1B). The washing experiment was conducted at ambient temperature using the remaining good kernels with maize to water ratio of 1:2. The washing procedure entailed a 5 min hand agitation, and for the longer time periods a 1 min agitation, prior to sequential collecting of duplicate subsamples at each time

point. The respective maize subsamples were dried as described above. The final sorted good kernel batch was used to investigate the effect of longer washing periods (15 and 24 h), with duplicate aliquots being withdrawn for fumonisin analyses as described above.

Determination of fumonisins in maize

The maize kernel samples were milled (Falling AB Stockholm, Sweden) prior to single fumonisin analyses, except for the infected/damaged kernel samples (n = 4), sorted from each batch, which were analysed in duplicate. In brief, according to the method of Sydenham et al. (1996), a milled maize sample (20 g) was homogenised in methanol-water and subsequent to centrifugation an aliquot of the supernatant was cleaned-up using strong anion exchange cartridges (Bond-Elut, Varian, Harbor City, CA, USA). The fumonisins were eluted with acetic acid-methanol, dried under nitrogen and stored at 4°C prior to analysis.

Chromatography

The preformed o-phthaldialdehyde derivatives were separated on a reversed-phase Luna 4µ C18 (2) (150 x 4.6 mm I.D.) column (Phenomenex, Torrance, CA, USA) and the isocratic mobile phase of methanol/0.1 M sodium dihydrogenphosphate (pH 3.35) (77:23) was pumped at a flow rate of 1 mL/min. The chromatographic system consisted of a Rheodyne 7725i injector (Cotati, CA, USA), Waters Model 515 solvent delivery system (Milford, MA, USA), Waters Breeze Chromatography Integration Software and Waters Fluorescence 474 detector (excitation - 335 nm and emission - 440 nm).

Results

Focus groups

Information was obtained from the focus groups regarding customary maize preparation prior to cooking. All the focus groups reported sorting the maize into good and mouldy maize cobs directly after the harvest. Mouldy cobs are discarded, with the exception of one group reporting the use of mouldy cobs for samp (dehulled stamped maize kernels) preparation. The good maize cobs are shelled either after sorting or just prior to food preparation. Thirty-five percent of the focus groups reported winnowing and removal of plant debris. Visibly mouldy kernels are removed by hand and the remaining “good” kernels are washed to dispose of any debris prior to cooking. Thirty percent of the focus groups reported a quick ambient water rinse, 35% wash the kernels for 3-8 min, 10% washed maize for 3-5 h and 25% used warm water. Wash water is discarded in the field (70%) or given to farm animals (30%).

Mycological analyses

The mycological results of 100 representative maize kernels are presented in Table 1. 17% of the maize kernels were infected with *F. verticillioides*. Other *Fusarium* species identified were *F. graminearum* (9%) *F. subglutinans* (5%) *F. anthophilum* (1%). Low levels of *Stenocarpella* species were observed. In contrast to previous studies conducted in other parts of the African continent, where *A. flavus* and the resultant aflatoxins are major contributors to the mycotoxin contamination load of the subsistence maize (Bankole et al., 2004; Kimanya et al., 2008b; Fandohan et al.,

2005; Wild and Gong, 2010), no *A. flavus* was detected in the maize collected for this study. Aflatoxin has only rarely been detected in South African commercial or subsistence maize (Shephard, 2003).

Chapter 6.1 - Table 1 Mycological assessment of home-grown maize

<i>Fusarium</i> species	Infected kernels (%)
<i>F. verticillioides</i>	17
<i>F. graminearum</i>	9
<i>F. subglutinans</i>	5
<i>F. anthophilum</i>	1
<i>Stenocarpella</i> species	
<i>S. maydis</i>	5
<i>S. macrospora</i>	1

Hand sorting of home-grown maize kernels

The mean fumonisin level of the unsorted home-grown maize samples (randomly selected) was 2.320 ± 1.155 mg/kg and the sorted good maize of all four batches was 0.68 ± 0.42 mg/kg (Table 2). The coefficient of variation (CV) of the unsorted ($n = 6$) and sorted good maize ($n = 16$) samples was 50% and 61%, respectively, showing the non-homogeneous distribution of fumonisin contamination in maize. The ratios of FB₁, FB₂ and FB₃ to (FB₁ + FB₂ + FB₃) were $71 \pm 2\%$, $21 \pm 2\%$, and $7 \pm 1\%$, respectively. An overall mean fumonisin reduction of $71 \pm 18\%$ was obtained by sorting out the visible/damage kernels. The mean weight of the infected/damaged kernels was 0.075 kg per 3 kg batch ($2.5 \pm 0.5\%$) with a mean fumonisin level of 53.7 ± 15.0 mg/kg.

Chapter 6.1 - Table 2 Fumonisin levels (mean \pm standard deviation) in maize kernels before and after sorting with the related reduction

Maize	n	Fumonisin (Range) (mg/kg)
Unsorted Maize	6	2.32 \pm 1.16 (0.82–4.08)
Sorted Good Maize	16	0.68 \pm 0.42 (0.16–1.69)
Infected/damaged kernels	7a	53.7 \pm 15.0 (37.5–76.2)
Reduction (%)		
Sorting		71 \pm 18 (27–93)

Means are not significantly different due to large standard deviations attributable to the non-homogenous distribution of fumonisin contamination

^aDuplicate samples from four batches, of which one sample were lost

Washing Experiments

Effect of Temperature

The sorted good kernel batches used for these experiments had a mean fumonisin level of 0.57 mg/kg compared to the means obtained for the 25°C and 40°C washes of 0.59 and 0.48 mg/kg, respectively (Table 3). The reduction in fumonisin level achieved by sorting for this kernel batch was 75 \pm 15%. An additional 4 \pm 6% reduction was obtained by washing the sorted good maize for 5 min at 40°C. No apparent reduction was achieved by washing the sorted good maize for 5 min at 25°C under these experimental conditions.

Effect of Wash Duration

The mean fumonisin level of the sorted good maize batch used in the hour experiment was 0.95 mg/kg (Table 3). Even under these conditions, where the sorting reduction was only 59% and with water to maize ratio of 2:1, the 5 min wash obtained only an additional 5 \pm 8% reduction. Therefore, no significant differences

Chapter 6.1 - Table 3 Fumonisin levels (mean \pm standard deviation) in sorted good kernels (SGK) and percentage reduction (mean \pm standard deviation) in the different washing experiments

	n	Fumonisin (mg/kg)	Reduction ^a (%)
Temperature Experiment			
SGK^b (no wash)	8	0.574 \pm 0.338	
5 min (25°C)	4	0.585 \pm 0.302	0 \pm 13
5 min (40°C)	4	0.476 \pm 0.144	4 \pm 6
Hour Experiment			
SGK (no wash)	4	0.950 \pm 0.669	
5 min	2	0.844 \pm 0.189	5 \pm 8
10 min	2	0.638 \pm 0.290	13 \pm 12
30 min	2	0.664 \pm 0.775	12 \pm 33
60 min	1 ^c	0.731	9
Day Experiment			
SGK (no wash)	4	0.623 \pm 0.148	
15 h	4	0.372 \pm 0.389	11 \pm 17
24 h	4	0.239 \pm 0.170	17 \pm 7

Means are not significantly different due to large standard deviations attributable to the non-homogenous distribution of fumonisin contamination

^aReduction based on mean unsorted level in homogeneity test

^bSorted good kernels (SGK)

^cLost one subsample

were seen between the two wash ratios as the fumonisin reductions obtained with the 5 min washes at 25°C and 40°C (maize to water ratio 1:1) were 0 \pm 13 and 4 \pm 6%. An additional 13 \pm 12% reduction was obtained after the 10 min wash, while longer wash periods, i.e. 30 and 60 min did not further improve the removal of fumonisins. The sorted good kernel batch used for the day experiment had a mean fumonisin level of 0.62 mg/kg and the reduction achieved by sorting was 73 \pm 6.0%. The additional 11 \pm 17% reduction obtained with the 15 h wash was similar to the

results obtained with the 10 min wash, whereas the additional 24 hour wash reduction was only slightly higher at $17 \pm 7\%$.

Discussion

This is the first study to optimise the reduction of fumonisin mycotoxins in home-grown maize based on customary methods of a rural population under laboratory-controlled conditions. Separating visibly infected from good kernels by sorting, followed by washing, are customary practices and practically implementable in the subsistence farming communities in the Centane magisterial district, South Africa.

Mycotoxin contamination of grains is characterised by the non-homogeneous distribution of the toxins among the grain kernels resulting in large inter-kernel variation. Even though great care was taken to thoroughly mix the large pool of maize kernels in this study, large variations in the fumonisins were observed in the unsorted and the treatment maize batches attributable to the non-homogeneous distribution of fumonisin contamination in maize kernels (Gilbert, 2002). In a specific study on the overall variance associated with determination of fumonisins in shelled maize, 61% of the total variance was associated with sampling, and only 22% with the actual analytical method (Whitaker et al., 1998). This observation was confirmed in the current study where the CV of the unsorted and sorted good maize samples randomly selected from a thoroughly mixed pool was 50% and 61%, respectively. Similar high CV values of up to 47% have been recorded in previous studies on maize batches (Canela et al., 1996). Nevertheless, the lack of homogeneity, which is

a major problem in sampling, represents an opportunity for reduction in contamination of the batch by the removal of only the most obviously infected or damaged kernels. This has also been recognised in industrial processing. A study of the cleaning of maize by screens and gravity separator during silo discharge indicated that removal of 7% of the maize by weight reduced the shelled maize fumonisin level from 7.90 mg/kg to 3.24 mg/kg, a reduction of 59% (Malone et al., 1998). Such a reduction method was the basis for prioritising visual sorting and the discarding of infected kernels as the first stage in achieving a practical and effective reduction strategy in subsistence maize. Fumonisin accumulates preferentially in the outer layers or pericarp of the maize kernel (Duncan and Howard, 2010). Steeping of maize kernels in water for periods between 6 and 48 hours showed that fumonisins can be leached from the pericarp layer (Canela et al., 1996). FB₁ levels in the steep water exceeded the levels in kernels after a period of 12-24 hours. In the industrial process of wet milling, 22% of the initial fumonisins were removed in the steep water during a laboratory scale wet milling experiment (Bennett and Richard, 1996).

The removal of infected/damaged kernels in the current study was very effective and the mean fumonisin reduction by sorting was $71 \pm 18\%$, while only 2.5% by weight of the maize was discarded. Hence a small sacrifice in food quantity substantially improved the quality of the maize kernels for food preparation. In terms of washing, an additional $13 \pm 12\%$ was achieved with the 10 min wash and although a 24-hour wash resulted in the greatest reduction (17%), the long wash duration would not be practical in the rural setting. No significant difference in fumonisin reduction between the maize to water ratio of 1:1 and 1:2 was observed. These results indicate that the greatest effect can be achieved by optimum sorting, and the additional effect of

washing was considerably smaller. This has the advantage in the rural communities that whatever maize dish is prepared, sorting will be appropriate.

The possible impact of a combined reduction (71% by sorting and an additional 13% by 10 min washing) in fumonisin levels on the estimated PDI values obtained in a previous study was re-estimated. The PDI of 8.7 $\mu\text{g/kg}$ body weight/day was based on the assumptions that a 60 kg adult consumed 0.456 kg maize-based food (dry weight)/day with a mean fumonisin level of 1.14 mg/kg (Shephard et al., 2007). An approximately 84% reduction in fumonisin contamination would potentially lower the fumonisin level to 0.183 mg/kg and thus reduce the estimated PDI to 1.4 $\mu\text{g/kg}$ body weight/day. Therefore the fumonisin reduction achieved in this study could potentially result in PDIs below the JEFCA PMTDI.

Based on these results and on local practices and practicalities the protocol that would be recommended to subsistence farmers consists of the removal of the infected/damaged kernels from the maize followed by a 10 min ambient temperature water wash with sufficient water to cover the maize. It remains necessary to emphasise that maize wash water needs to be discarded, rather than used for further cooking. The use of mouldy maize on occasion is a cause for concern as it could dramatically increase the PDI of fumonisin and the associated health risk in the subsistence farming community. This practical culturally acceptable method of sorting maize by removal of the infected/damaged kernels and the subsequent washing of the good maize kernels effectively reduced fumonisin contamination of home-grown maize. The recommendations based on these experiments will be

tested in an intervention study conducted in the high oesophageal cancer incidence area of Centane magisterial district, South Africa.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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6.2

Implementation of simple intervention methods to reduce fumonisin exposure in a subsistence maize farming community of South Africa

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Abstract

High oesophageal cancer incidence rates have been associated with home-grown maize highly contaminated with fumonisins in the Centane magisterial area, South Africa. The aim of this study was to implement a simple intervention method to reduce fumonisin exposure in a subsistence farming community. The hand-sorting and washing procedures based on the traditional maize-based food preparation practices were previously customised under laboratory-controlled conditions. Home-grown maize and maize-based porridge collected at baseline were analysed for fumonisin B₁, B₂ and B₃. The geometric mean (95% confidence interval) of the fumonisin contamination in the home-grown maize at baseline was 1.67 (1.21–2.32) mg/kg and 1.24 (0.75–2.04) mg/kg (dry weight) in the porridge. Fumonisin exposure was based on individual stiff porridge consumption and the specific fumonisin levels in the porridge (dry weight) consumed. Porridge (dry weight) consumption at baseline was 0.34 kg/day and fumonisin exposure was 6.73 (3.90–11.6) µg/kg body weight/day. Female participants (n=22) were trained to recognise and remove visibly infected/damaged kernels and to wash the remaining maize kernels. The discarded kernels represented 3.9% by weight and the fumonisins varied from 17.1–76.9 mg/kg. The customised hand-sorting and washing procedures reduced fumonisin contamination in the maize and porridge by 84% and 65%, respectively. The intervention reduced fumonisin exposure by 62% to 2.55 (1.94–3.35) µg/kg body weight/day. This simple intervention method has great potential to improve food safety and health in subsistence farming communities consuming fumonisin contaminated maize as their staple diet.

Keywords: fumonisin, exposure, home-grown maize, reduction, intervention, subsistence farmers, South Africa

Introduction

Maize is a dietary staple in large parts of Africa and often consumed almost to the exclusion of all other food commodities (Shephard et al. 2008). This total reliance on maize leads to chronic exposure to fumonisins and often to other mycotoxins co-contaminating maize, e.g. aflatoxin (Fandohan et al. 2005). As subsistence communities would not benefit from international or national regulations to control fumonisin exposure, alternative measures to reduce fumonisin contamination in home-grown maize are required (Gelderblom et al. 2008; Shephard et al. 2008; Wild and Gong 2010).

Consumption of fumonisin contaminated maize by subsistence farmers in high oesophageal cancer incidence areas has been reported in several countries e.g., Italy, China, USA and Brazil (Doko and Visconti 1994; Marasas 2001; Sun et al. 2007; Sydenham et al. 1991; Van der Westhuizen et al. 2003). Primary liver cancers in China and increased occurrence of neural tube defects in Mexico have also been associated with high fumonisin exposure (Marasas et al. 2004, Sun et al. 2007). As fumonisins are ubiquitously present in maize-based food, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) has determined a group provisional maximum tolerable daily intake (PMTDI) for fumonisin B₁, B₂ and B₃, (FB₁, FB₂ and FB₃) alone or in combination, of 2 µg/kg body weight (bw)/day (Shephard et al. 1996; Bolger et al. 2001).

Fumonisin contamination of maize is non-homogenous and can be effectively reduced by the removal of visibly infected kernels (Afolabi et al. 2006; Fandohan et

al. 2005; L van der Westhuizen et al. unpublished data). This is of particular importance in rural subsistence communities with low socio-economic status reliant on a mono cereal diet for most of the year. These communities are the most vulnerable to the toxic and carcinogenic effects of mycotoxins and therefore intervention methods should be simple, cost effective and aligned with the local customs (Desjardins et al. 2000). A Nigerian study on sorting of maize by subsistence farmers into visibly poor and good quality samples reported reduction in fumonisin exposure provided that poor quality maize is not consumed (Afolabi et al. 2006). A reduction of up to 87% in fumonisins was reported in naturally contaminated maize processed according to various traditional practices, such as sorting, winnowing and washing in Benin, West Africa (Fandohan et al. 2005). Another study conducted in Benin recommended that subsistence farmers use appropriate shelling and dehulling methods to reduce fumonisin in contaminated maize (Fandohan et al. 2006). Improper shelling methods, causing kernel damage, and storage under humid conditions resulted in increased levels of fumonisin. As fumonisin levels are higher in the pericarp of the maize kernel, different mechanical dehulling methods reduced fumonisin contamination by 57–65% (Fandohan et al. 2006; Sydenham et al. 1995). Selecting specific maize hybrids, fertilizing and sorting of maize prior to storage were reported to reduce fumonisin contamination of subsistence grown maize in Tanzania (Kimanya et al. 2009).

Although these fumonisin studies achieved reduction in maize by different food preparation procedures, agricultural practices, sorting, mechanical shelling and dehulling, no intervention was implemented (Afolabi et al. 2006; Fandohan et al. 2005; 2006; Kimanya et al. 2009). An aflatoxin intervention study, conducted in a

subsistence farming community, reported that improved sorting, drying and storage conditions substantially reduced the levels in groundnuts and exposure in individuals assessed with the aflatoxin-albumin biomarker (Turner et al. 2005). Studies conducted in the Centane magisterial district of the Eastern Cape Province, South Africa, have reported some of the highest rates for oesophageal cancer, associated with high levels of fumonisin-contaminated home-grown maize (Marasas 2001; Gelderblom et al. 2008). Recently the customary practices of hand-sorting and washing kernels, prior to food preparation, optimised under laboratory-controlled conditions achieved a 73% reduction of fumonisins in home-grown maize (L van der Westhuizen et al. unpublished data).

The aim of this study was to implement and evaluate the effectiveness of this customised simple intervention method – hand-sorting and washing of maize kernels – to reduce fumonisin exposure in a subsistence-farming community residing in an area of South Africa with high oesophageal cancer incidence.

Material and Methods

Chemicals

Fumonisin (FB₁, FB₂ and FB₃) standards were isolated and purified according to the method of Cawood et al. (1991). All other chemicals and solvents were analytical grade from Merck (Darmstadt, Germany).

Participants

The study was approved by the Ethics Committee of the Medical Research Council of South Africa. Following informed consent apparently healthy females (n = 22) who prepared maize-based meals from home-grown maize were recruited from the Centane magisterial district of the Eastern Cape Province.

Baseline phase of the field study (maize and food collection)

Participants were requested to prepare maize-based food (stiff porridge) from maize following their customary practices and to consume approximately two x 0.5 kg stiff porridge portions per day for two consecutive days. Twenty-four-hour dietary recall questionnaires, utilising full-scale photographs of small, medium and large stiff porridge portions, were completed in the local vernacular (isiXhosa) to establish compliance and determine the actual quantity of maize intake. Individual maize intakes (dry weight, g/day) were estimated using the local maize porridge recipes and by assigning specific weights to the small, medium and large stiff porridge portions as reportedly consumed by the participants (Burger et al. 2010). Stiff porridge samples (~ 0.5 kg) were collected on the two consecutive days and stored at -20°C prior to fumonisin B₁, B₂ and B₃ analyses. Home-grown maize kernel samples (n = 22, ~ 5 kg) used for porridge preparation were collected from each participant on the first day of their participation. A subsample (~ 0.5 kg) of each maize collection was retained for fumonisin analyses. The remaining kernels were pooled, thoroughly mixed, divided into four kg batches (n = 22) and set aside for the intervention study. An additional subsample (4 kg) was retained for duplicate fumonisin analyses.

Intervention phase of the field study (maize and food collection)

The participants were trained by the field workers, by demonstrating the removal of infected kernels with the aid of photographs that visually illustrated infected and damaged maize kernels. The photographs (A4 size) depicted the infected kernels optimally removed by a plant pathologist under laboratory conditions. Although it is not possible to visually recognise fumonisin-contaminated kernels from the systemic, symptomless mode of association between *Fusarium verticillioides* and the maize plant, higher fumonisin levels usually occur in symptomatic kernels (Munkvold and Desjardins 1997; Rheeder et al. 1992). Furthermore, *F. subglutinans* symptomology on maize is similar to *F. verticillioides* and to some degree also *F. proliferatum*, but produces little or no fumonisin (Reynoso et al. 2004). Following the training session, each participant sorted an individual 4 kg maize kernel batch under the supervision of the field workers. The sorting should be incorporated into the food preparation and need not take longer than 10 minutes. On completion of the sorting by the participants, the infected and damaged maize kernels of each batch were weighed separately and retained for fumonisin analyses.

After sorting, the fieldworkers demonstrated the 10 minute maize washing procedure (sufficient water to cover kernels), consisting of a five minute hand agitation followed by a one minute agitation immediately prior to the 10 minute end point. Each participant washed their remaining “good” kernels under the supervision of the field workers. Subsamples (0.5 kg) of each sorted and washed sample were dried at ambient temperature prior to fumonisin analyses. The remaining kernels of each sample were pooled, dried at ambient temperature for two days, thoroughly mixed, milled and stored. A subsample (5 kg) was retained for duplicate fumonisin analyses.

The fieldworkers prepared the traditional stiff porridge from the milled, sorted and washed maize on two consecutive days and the participants consumed at a communal 'food party' a weighed portion (0.5 kg) each. Another 0.5 kg portion was supplied to each participant for a subsequent meal at a later stage on the same day. Subsamples of the porridge, prepared from the pooled milled samples, were collected on the consecutive days and stored at -20°C prior to analyses. A 24-hour dietary recall questionnaire was completed on the two consecutive days following the food parties to assess the porridge intake (dry weight).

Fumonisin analyses

The maize kernel [baseline unsorted (n = 22), sorted and washed good (n = 21*) and sorted visibly infected/damaged (n = 21) kernels] were ground and analysed for fumonisin according to the method of Shephard et al. (2002) with minor modifications. [*One of the participants recruited at the baseline phase of the study did not participate in the intervention phase of the study]. The pooled and milled maize from the sorted and washed good kernels, which had been retained for the intervention study, was also analysed. In brief, a 20 g maize subsample was extracted with 100 mL methanol:water (3:1) by shaking for 1 hour, centrifuged and filtered. The pH was adjusted and an aliquot (10 mL) of each extract was cleaned-up on a strong anion exchange (SAX) solid phase extraction cartridge. The fumonisins were eluted with acetic acid-methanol, dried under nitrogen and stored at 4°C prior to analysis. The results were expressed as mean mg total fumonisins (FB₁ + FB₂ + FB₃)/kg maize.

Maize-based food (stiff porridge)

The fumonisin content of the maize porridge was determined according to the method of Shephard et al. (2002) with minor modifications. In brief, the moisture content of the maize porridge was first measured by drying a sample (30 g) to constant weight in an oven at 60 °C for 48 hours. The moisture content was obtained from a separate subsample for each porridge sample. Prior to fumonisin extraction of the maize porridge (40 g), water was added to obtain a final volume of 30 mL in the sample and extracted after the addition of 70 mL methanol. Each porridge/water/methanol mixture was blended for 30 seconds in a liquidiser to a homogeneous consistency and was then mechanically shaken for one hour. The remainder of the extraction and fumonisin analyses were conducted as described above for the maize kernels. The results were expressed as mean mg total fumonisins/kg porridge (dry weight).

Chromatography

Sample residues were redissolved in 200 µL methanol and aliquots derivatised with *o*-phthaldialdehyde. The derivatised-fumonisin were separated on a Phenomenex (Torrance, CA) Luna 5 µm C18(2) column (75 x 4.6 mm I.D.) using a mobile phase of methanol 0.1 M sodium phosphate buffer (78:22, pH 3.35) pumped at a flow rate of 1 mL/min. Quantification was by peak area comparison with a similarly derivatised standard. The chromatographic system consisted of a Rheodyne 7725i injector (Cotati, CA, USA), Waters Model 515 solvent delivery system (Milford, MA, USA), Waters Breeze Chromatography Integration Software and Waters Fluorescence 474 detector (excitation - 335 nm and emission - 440 nm).

Fumonisin exposure

Fumonisin exposures were assessed on an individual basis as the total fumonisin level in the stiff porridge (dry weight) consumed by each participant during the baseline and intervention phases of the study. Porridge (dry weight) consumption was assessed by 24-hour dietary recall questionnaires.

Statistical analysis

The fumonisin data were tested for normality and equality of variances, with natural log transformation of all variables. The fumonisin data before and after intervention were compared using a t-test. The STATA version 9 software package (StataCorp, Texas, USA) was used for statistical analysis. Means were expressed geometrically (95% confidence interval).

Results

Participants

The mean age of the participants was 45 years with a range of 18–70 years and their mean body weight was 65 kg (57–73 kg) with a range of 47–127 kg (Results not shown). The participant compliance was good as 21/22 completed the baseline phase and 20/22 completed the intervention phase of the field study.

Fumonisin in maize

The individual home-grown maize (n = 22) collected at baseline had a mean fumonisin level of 1.67 (1.21–2.32) mg/kg with a range of 0.36–4.90 mg/kg and a

coefficient of variation (CV) of 65%, whereas the pooled sample of the home-grown maize was 1.32 mg/kg (Table 1). The mean fumonisin level of the individual sorted and washed maize kernels ($n = 21$) was 0.27 (0.20–0.36) mg/kg with a range of 0.09–0.70 mg/kg and a CV of 56% compared, to 0.26 mg/kg in the pooled, sorted and washed maize sample. The participants achieved 84% mean reduction by hand-sorting and washing the maize kernel batches, whereas the individual variation in reduction was 13%. The individual batches ($n = 21$) of the discarded visibly infected/damaged kernels had a mean weight of 0.15 (0.13–0.18) kg representing 3.9 (3.2–4.6) % of the initial 4 kg maize batches. The infected and damaged maize kernels removed by each participant had a mean fumonisin level of 34.6 (27.8–41.3) mg/kg and ranged from 17.0–76.9 mg/kg with a CV of 42%.

Fumonisin in maize-based food (stiff porridge)

The moisture content obtained separately for each porridge sample ($n = 22$) was [68.2 (66.7–69.8)%]. Analytical results were expressed as mg total fumonisin/kg porridge (dry weight). The fumonisin level in the stiff porridge ($n = 22$) collected at baseline was 1.24 (0.75–2.04) mg/kg with a range of 0.08–6.58 mg/kg and a CV of 95%. The porridge prepared by fieldworkers for the intervention phase of the study had a mean fumonisin level of 0.43 (0.38–0.50) mg/kg and a CV of 32%. The intervention procedure reduced the fumonisin level in traditional stiff porridge by 65%.

Fumonisin exposure

The mean actual on plate stiff porridge consumption as recorded from the 24-hour dietary recall questionnaires at baseline was 1.06 (0.92–1.12) kg/day and following

Chapter 6.2 - Table 1 Mean porridge (dry weight) consumption, fumonisin levels in maize and food as well as fumonisin exposure in the Centane area of the Transkei region in South Africa

	Porridge Consumption kg (dry weight)/day	Fumonisin in Maize mg/kg	Fumonisin in Porridge mg/kg (dry weight)	Fumonisin PDI µg/kg b.w./day	PDI > PMTDI
Baseline	0.34a (0.28–0.40)	1.67a (1.21–2.32)	1.24a (0.75–2.04)	6.73a (3.90–11.62)	18/21
Intervention	0.38a (0.31–0.47)	0.27b (0.20–0.36)	0.43b (0.38–0.50)	2.55b (1.94–3.35)	13/20
Reduction		84%	65%	62%	

Notes: Porridge consumption = dry weight based on 24-h dietary recall assessment.

Fumonisin = $FB_1 + FB_2 + FB_3$

Fumonisin in porridge = fumonisin levels in stiff porridge (dry weight).

PDI = probable daily intake of fumonisins.

Fumonisin PDI = based on fumonisin levels in stiff porridge (dry weight) and stiff porridge consumption (dry weight).

bw = body weight.

PMTDI = provisional maximum tolerable daily intake of 2 µg fumonisin/kg body weight/day (JECFA).

Values = geometrical mean (95% confidence interval).

Means are significantly ($p < 0.05$) different when followed by different letters within columns.

*Good compliance, 21/22 recruited participants completed baseline and 20/22 completed intervention phase of the field study.

intervention 1.09 (0.87–1.12) kg/day. The mean stiff porridge (dry weight consumption at baseline was 0.34 (0.28–0.40) kg/day compared to 0.38 (0.31–0.47) kg/day following intervention. Mean probable daily intake (PDI) for fumonisins at baseline was 6.73 (3.90–11.6) µg/kg body weight/day compared to 2.55 (1.94–3.35) µg/kg body weight/day following intervention. The reduction in fumonisin exposure achieved by preparing porridge from sorted and washed maize kernels was 62%. At baseline 18/21 participants had PDI's above the PMTDI determined by JECFA (2 µg/kg body weight/day) compared to 13/20 following intervention.

Discussion

This is the first intervention study to reduce fumonisin exposure based on the customary maize-based food preparation practices in a subsistence farming community. The simple two-step intervention method (sorting and washing) prior to the actual cooking of the maize did not prolong the food preparation time unduly and would thus be culturally sustainable. During the intervention, fieldworkers trained the participants to identify the infected/damaged maize kernels to ensure proper selection for removal and to follow the correct washing procedures. When taking into consideration the non-homogeneous nature of the fumonisin contamination, the 13% variation in the reduction by each participant show the effective implementation of the hand-sorting and washing procedure. The simple and culturally acceptable intervention method reduced fumonisin contamination by 84%; surpassing the 73% reduction achieved under laboratory-controlled conditions (L van der Westhuizen et al. unpublished data).

The reduction achieved by visual means is comparable with a mean fumonisin reduction of 82% obtained by mechanical means with near-infrared spectroscopy where the discarded infected/damaged kernels comprised 5% by weight (Pearson et al. 2004). Although the process of sorting and washing of maize is part of the customary cooking procedure, the 65% reduction of fumonisins in the porridge during intervention is indicative of the improvement that can be achieved. Therefore it is imperative that the participants understand the importance of the infected/damaged kernel removal and the accurate identification in order to achieve maximum benefit. For example, in a Tanzanian study the customary sorting of maize by removal of infected/damaged maize by subsistence farmers, as opposed to the optimized method introduced in the current study in the context of educational training, was not effective in minimizing fumonisin exposure (Kimanya et al. 2008). In the present study fumonisin levels were lower in the porridge (1.24 mg/kg) than in the home-grown maize (1.67 mg/kg) at baseline as it was not prepared from the same pool of maize. In addition the fumonisins levels in the home-grown maize collected from each household's supply ranged from 1.21 to 2.32 mg/kg. However, following intervention the mean fumonisin level in the porridge (0.43 mg/kg) was higher than in the sorted and washed maize (0.27 mg/kg) from which it was prepared. This could be ascribed to lower recoveries in maize than in porridge and to the non-homogenous nature of fumonisin kernel contamination (Shephard et al. 2002; Whitaker et al. 1998).

The mean stiff porridge (wet weight) consumption obtained from the 24-hour dietary recall questionnaires at baseline was 1.06 (0.92–1.12) kg/day and during intervention was 1.09 (0.87–1.12) kg/day. This confirmed that the participants

complied with the requested consumption of the two x 0.5 kg stiff porridge during the baseline phase and for the second portion of porridge supplied during the intervention phase of the study. The stiff porridge (dry weight) consumption at baseline (0.34 kg/day) and during intervention (0.38 kg/day) were comparable to maize-based food (dry weight) consumption (0.43 kg/day for females in Centane) assessed with weighed food records from a previous study (Shephard et al. 2007). In the present study the PDI (6.74 µg/kg body weight/day) at baseline was based on individual stiff porridge consumption and the specific fumonisin levels in the porridge (dry weight) consumed. PDIs estimated from various assumptions in previous investigations in Centane were 8.15 µg/kg body weight/day for females (Shephard et al. 2007) and 4.41 and 6.69 µg/kg body weight/day for all participants during the 1997 and 2000 harvest seasons, respectively (Van der Westhuizen et al. 2008). These studies highlighted that the mean exposure to fumonisin in this region exceeded the JEFCA PMTDI (Bolger et al. 2001). In the current study 18/21 participants at baseline were exposed to fumonisin levels above the PMTDI (2 µg/kg body weight/day) set by JECFA. The 62% reduction in fumonisin exposure (PDI) achieved with the hand-sorting and washing procedure resulted in a slightly decreased number of participants exposed to levels above the PMTDI despite the fact that the daily exposure was radically reduced. This was due to the high consumption of a single food commodity and additional measures to further reduce fumonisin exposure should be considered. Preliminary investigation into weaning food during the current study showed that it would also have reduced fumonisin levels as the only difference is that larger amounts of water are utilized during the weaning food preparation.

The customised hand-sorting and washing of fumonisin contaminated-maize by the subsistence farmers to reduce fumonisin exposure was implemented effectively. The possible impact of the intervention on human health is difficult to assess without epidemiological studies utilising a reliable biomarker of exposure. However, this simple and culturally acceptable method has the potential to improve food safety and health in these subsistence farming communities, exposed to unsafe levels of fumonisin.

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6.3

Fumonisin B₁ as a urinary biomarker of exposure in a maize intervention study among South African subsistence farmers

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Abstract

Background: The consumption of maize highly contaminated with carcinogenic fumonisins has been linked to high oesophageal cancer rates. The aim of this study was to validate a urinary fumonisin B₁ (UFB₁) biomarker as a measure of fumonisin exposure and to investigate the reduction in exposure following a simple and culturally acceptable intervention.

Methods: At baseline home-grown maize, maize-based porridge and first void urine samples were collected from female participants (n=22), following their traditional food practices in Centane, South Africa. During intervention the participants were trained to recognise and remove visibly infected kernels, and to wash the remaining kernels. Participants consumed the porridge prepared from the sorted and washed maize on each day of the two-day intervention. Porridge, maize and urine samples were collected for FB₁ analyses.

Results: The geometric mean (95% confidence interval) for FB₁ exposure based on porridge (dry weight) consumption at baseline and following intervention was 4.84 (2.87–8.14) and 1.87 (1.40–2.51) µg FB₁/kg body weight/day, respectively, (62% reduction, p<0.05). UFB₁C, UFB₁ normalized for creatinine, was reduced from 470 (295–750) at baseline to 279 (202–386) pg/mg creatinine following intervention (41% reduction, p=0.06). The UFB₁C biomarker was positively correlated with FB₁ intake at the individual level (r=0.4972, p<0.01). Urinary excretion of FB₁ was estimated to be 0.075% (0.054%–0.104%) of the FB₁ intake.

Conclusion: UFB₁ reflects individual FB₁ exposure and thus represents a valuable biomarker for future fumonisin risk assessment.

Impact: The simple intervention method, hand sorting and washing, could positively impact on food safety and health in communities exposed to fumonisins.

Introduction

Fumonisin is a group of mycotoxins; mainly produced by *Fusarium* spp. as secondary metabolites, of which fumonisin B₁, B₂ and B₃ (FB₁, FB₂ and FB₃) are the most prevalent (1). Fumonisin is carcinogenic to rodents, have been associated with oesophageal and liver cancer in subsistence maize farming communities around the world, as well as occurrence of neural tube defects (2, 3, 4, 5). There are numerous fumonisin analogues of which FB₁ is the most abundant naturally occurring fumonisin on maize and maize-based food world-wide and was classified as “possibly carcinogenic to humans” by the International Agency for Research on Cancer (6). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has determined a group provisional maximum tolerable daily intake (PMTDI) for FB₁, FB₂ and FB₃, alone or in combination, of 2 µg/kg body weight/day (7).

In many Sub-Saharan countries, where both maize contamination and maize consumption are high, regulatory mechanisms to control mycotoxin levels, including fumonisin, are either lacking or are not enforced (8). Therefore reducing exposure levels by intervention, specifically those based on simple low-cost measures acceptable to these communities, becomes critical to protect the population at greatest risk (9). Such an approach gave promising results with respect to aflatoxins in groundnuts (10).

Conventional assessment of mycotoxin exposure, probable daily intake (PDI), is based on the amount of food consumed and the contamination level of the food (11). Accuracy of the PDI can be improved by determining food consumption and the level

of the mycotoxin in the food on an individual basis (12). The quality of food consumption information in the subsistence community setting depends on the validity of the assessment methods and measuring of food on the plate prior to and after food consumption, a labour-intensive activity (11). Utilization of validated biomarkers of exposure, taking advantage of measuring individual internal dose, would not require measurement of consumption or contamination level of the food and thus promises to be more accurate than estimations by other assessment methods (13). Aflatoxin, deoxynivalenol and ochratoxin A urinary biomarkers, as well as the aflatoxin-albumin adduct in blood, have been implemented in human studies (14–16). The aflatoxin biomarker applications in human health and intervention studies have demonstrated the value of mycotoxin-related biomarkers (13).

Various animal studies have successfully investigated the sphinganine/ sphingosine ratio as a biomarker of fumonisin exposure (11, 17). The sphingolipid bases, sphinganine and sphingosine, as well as their ratio, have also been investigated in several human studies in blood and urine, but could not be correlated with fumonisin exposure (11, 12, 18). A recent study has reported a HPLC-MS method for urinary FB₁ (UFB₁) which was sufficiently sensitive to be positively correlated with fumonisin exposure in a Mexican population consuming different amounts of maize-based tortillas (19).

In a previous study, customary sorting and washing of maize kernels as food preparation procedures were optimized under laboratory-controlled conditions to achieve optimal fumonisin reduction in contaminated home-grown maize (20). These simple and culturally acceptable food preparation practices reduced fumonisin

exposure as assessed by food intake profiles and fumonisin food analysis in a subsistence farming community residing in a high oesophageal cancer incidence area (21). The aim of this study was to validate the UFB₁ biomarker and confirm the reduction in fumonisin exposure at an individual level.

Material and methods

Participants

The study was approved by the Ethics Committee of the Medical Research Council of South Africa. Following informed consent, apparently healthy females (n=22) aged between 20 and 70 who prepared maize-based meals from home-grown maize, were recruited from the Centane magisterial district, Eastern Cape Province, South Africa.

Baseline phase of the field study

At baseline, participants consumed their customarily prepared maize-based food (~0.5 kg porridge) twice daily for two consecutive days (Table 1). Porridge samples (~0.5 kg) were collected on both days for FB₁ analyses. Morning first void urine samples were collected from each participant on the subsequent days for FB₁ biomarker analysis. The first-void urine collections were done approximately 12 hours after the participants consumed the last porridge meal. Dietary recall questionnaires were recorded to determine maize intake (dry weight) in the 24 h prior to urine collection (21). Home-grown maize samples (~5 kg) were collected from each participant of which subsamples (~0.5 kg) were retained for FB₁ analyses.

The remaining kernels were pooled, thoroughly mixed, divided into 4 kg batches (n=22) and set aside for the intervention study.

Chapter 6.3 - Table 1 Both the baseline and intervention phases of the study were conducted over 3 consecutive days for each participant. Morning first void urine samples were collected from the participant individually on each day following the twice daily consumption of the porridge. The training was conducted following the completion of the baseline phase preceding the intervention phase of the study.

Baseline			Training	Intervention		
Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Consume porridge ↓	Consume porridge ↓ Collect first void urine	Collect first void urine	Sorting/ washing of maize Collect sorted/ washed maize	Consume porridge ↓	Consume porridge ↓ Collect first void urine	Collect first void urine

Intervention phase of the field study

Participants were trained to recognise infected kernels (most likely to be contaminated) and on the washing procedure (10 min) for the remaining maize kernels. Following training each participant first removed visibly infected kernels and subjected the remaining maize kernels to a washing step. Subsamples of the sorted and washed maize of each participant were collected for FB₁ analysis and the remainder of the maize kernels pooled for porridge preparation. On the first day of the intervention phase porridge was prepared and weighed as 0.5 kg portions which

were consumed as two separate meals (midday and evening) by the participants. First void urine was collected on the following morning and 24-h dietary recall questionnaires completed as described above. The procedure was repeated on the second day of the intervention. All the maize and porridge samples were stored at 4°C and -20°C, respectively prior to FB₁ analysis at the PROMEC Unit. Urine samples were stored at -20°C and sent on dry ice for FB₁ analysis at the University of Leeds.

Analyses

The maize and porridge samples were analysed for FB₁ following solid phase extraction clean-up, derivatization with *o*-phthaldialdehyde and detected by fluorescence HPLC (21, 22). The urine samples were cleaned-up by solid phase extraction and analysed for FB₁ by HPLC-MS with a limit of detection (LOD) of 20 pg/mL (19). Urinary creatinine was determined according to the alkaline-picrate method (23) with minor modifications to adapt to a 96-well plate format. Inter-individual variation in urine concentration was normalized by utilisation of urinary creatinine. The biomarker data are presented as both pg FB₁/mL urine (UFB₁) and pg FB₁/mg creatinine (UFB₁C).

Probable daily intake (PDI) assessment

Porridge (dry weight) consumption was assessed by 24-h dietary recall questionnaires utilising full-scale photographs of small, medium and large porridge portions. Individual maize intakes (dry weight, g/day) were estimated using the local maize porridge recipes and by assigning specific weights to the portions sizes (21). Individual fumonisin PDI was assessed as FB₁ level in the porridge (dry weight)

consumed by each participant during the baseline and intervention phases of the study.

FB₁ excretion in urine

The 24-h urine output was estimated by assuming that the first void urine volume represented an 8-h collection. The urinary FB₁ excretion was calculated as follows:

$$\text{Excretion (\%)} = \frac{\text{UFB}_1 \text{ (pg/mL)} \times \text{8-h urine output (mL/day)} \times 3}{\text{PDI } (\mu\text{g/kg body weight/day}) \times \text{body weight (kg)} \times 10^6} \times 100$$

Statistical analysis

Individual FB₁ PDI and UFB₁ were assessed as the mean of two days. UFB₁C data was not normally distributed and was natural log transformed to facilitate data analysis. Mean FB₁ levels in urine and maize were expressed geometrically (95% confidence interval) unless otherwise stated. Student's *t* test was used to compare the FB₁ exposure at baseline and following intervention. Correlation and regression analyses were performed to examine the association between the PDI and UFB₁ or UFB₁C level. Urine samples below the LOD were assigned a value of half the LOD. Multiple linear regression modelling was used to investigate the independent contribution of FB₁ PDI and age to the biomarker levels. STATA9.0 (STATA corp, Texas, USA) and Number Cruncher Statistical Systems (NCSS) 2004 Dawson Edition (NCSS, Kaysville, Utah) were used for the analysis.

Results

Participants

The mean age of the participants was 42 (range 20–70) years, their mean body weight was 65 (47–127) kg and their mean maize consumption (dry weight) at baseline was 0.34 (0.28–0.40) kg/day and following intervention it was 0.38 (0.31–0.47) kg/day as reported in the previous study (20). The mean FB₁ level in home-grown maize at baseline [1.16 (0.84–1.56) mg/kg] was significantly reduced (84%, $p<0.05$) following the sorting and washing of the kernels during intervention [0.19 (0.14–0.25) mg/kg]. The FB₁ represented 72% of the total fumonisins (FB₁ + FB₂ + FB₃) in the maize (FB₂ and FB₃ levels were reported in a previous study) (20). The porridge at baseline had a mean FB₁ of 0.89 (0.55–1.44) mg/kg compared to 0.32 (0.27–0.37) mg/kg following intervention (64% reduction, $p<0.05$) (Table 2).

PDI assessment

The mean PDI of FB₁ at baseline was 4.84 (2.87–8.14) µg/kg body weight/day and following intervention the PDI was significantly reduced (62%, $p<0.05$) to 1.87 (1.40–2.51) µg/kg body weight/day (Table 2). The mean FB₁ exposure at baseline exceeded the JECFA recommended PMTDI (2 µg/kg body weight/day), and was below this PMTDI following intervention. Before intervention 15/21 (71%) participants had PDIs exceeding the recommended PMTDI, while this frequency was reduced to 10/19 (53%) following intervention (chi square=1.5038, $p=0.22$). However, FB₁

Chapter 6.3 - Table 2 The geometric means (95% confidence limits) of FB₁ levels in porridge and urine, as well as PDI in Centane, a rural area from the Eastern Cape Province of South Africa.

	FB ₁ Porridge (mg/kg)	FB ₁ PDI [#] (µg/kg b.w./day)	UFB ₁ (pg/mL)	UFB ₁ C [†] (pg/mg creatinine)
Baseline	0.89a (0.55–1.44)	4.84a (2.87–8.14)	225a (144–350)	470a (295–750)
Intervention	0.32b (0.27–0.37)	1.87b (1.40–2.51)	109b (85–138)	279a (202–386)
Reduction	64%	62%	52%	41%

[#]PDI based on FB₁ levels in porridge (dry weight) consumed as assessed with 24-h dietary recall questionnaires

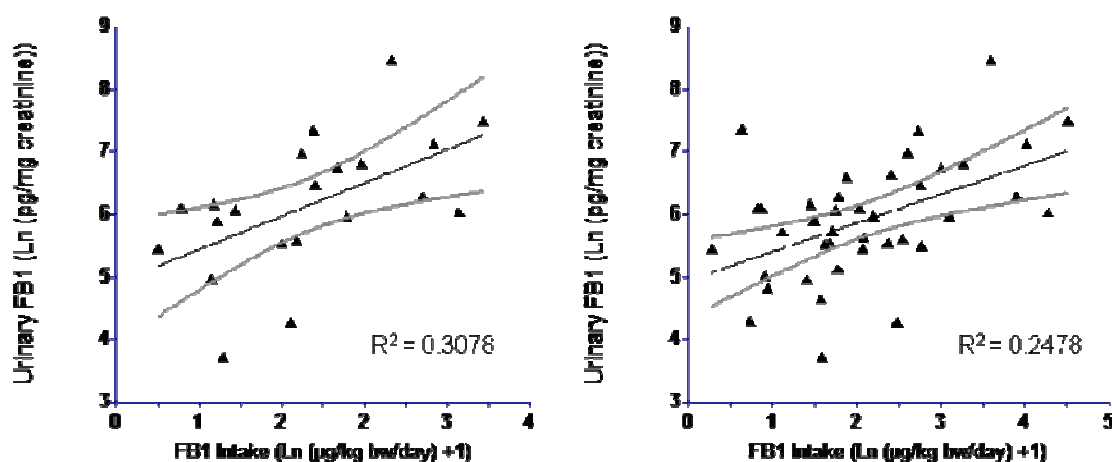
[†]UFB₁ was normalized with urinary creatinine

Means are significantly ($p < 0.05$) different within columns when followed by different letters

Urine

At baseline 43/44 (98%) and following intervention 40/42 (96%) of the urine samples had FB₁ above the LOD. Following intervention the mean UFB₁ was reduced from 225 (144–350) pg/mL to 109 (85–138) pg/mL, a 52% reduction ($p=0.02$) (Table 2). Following normalization with urinary creatinine, UFB₁C at baseline and following intervention was 470 (295–750) pg/mg and 279 (202–386) pg/mg creatinine, respectively, (41% reduction, $p=0.06$). Individual FB₁ intake to UFB₁C at baseline and intervention combined is significantly correlated ($r=0.4972$, $p<0.01$). In addition the linear regression relationship between FB₁ intake and UFB₁C, at baseline only and at baseline and intervention combined, is shown in Figure 1. There is a significant positive correlation between FB₁ intake and UFB₁C level (regression

coefficient 0.4154, $p < 0.01$). However, the multiple regression model is better fitted when age, a positive contributor (regression coefficient 0.0227, $p < 0.01$), is introduced as the older women were apparently exposed to higher FB_1 levels.



Chapter 6.3 - Figure 1 The relationship between UFB_1C and FB_1 intake (natural log transformed data) at baseline (left) and at baseline and intervention combined (right) is shown. The lines show the predicted linear regression for UFB_1C and the grey curves indicate the 95% confidence limits.

FB_1 excretion in urine

The urine output as well as the urinary excretion was similar at baseline and following intervention. The estimated mean 24-h urine output at baseline and during intervention combined was 933 (793–1,099) mL/day. The mean percentage FB_1 excreted in urine based on FB_1 intake and the estimated mean daily urine output was 0.075%/day (0.054%/day to 0.104%/day).

Discussion

This is the first published study describing a quantitative correlation between a fumonisin biomarker and intake of this mycotoxin measured at the individual level. The investigation provides further validation following a previous report showing a positive correlation between the FB₁ urinary biomarker and different levels of maize-based tortilla consumption in a Mexican population (19). In addition, the current investigation applied an optimized sorting and washing method to maize which reduced fumonisin exposure in a subsistence farming community as previously assessed by food analysis and food intake data (21). The biomarker was well correlated with FB₁ exposure at the individual level and confirmed the efficacy of this simple and culturally acceptable intervention method.

Surveys previously conducted in subsistence farming communities in Africa have shown that mycotoxin contamination, such as fumonisin and aflatoxin, can be reduced by hand-sorting and/or washing of maize kernels (24, 25). Featured in this paper and our previous publication (21) the first intervention study to reduce fumonisin exposure by implementing these practices has been conducted recently in a rural subsistence-farming community in South Africa. The customary sorting and washing of maize kernels as food preparation procedures were optimized under laboratory-controlled conditions. The participants were trained to apply the simple two-step method by identifying the infected maize kernels to ensure proper selection for removal and to follow the correct washing procedures. The intervention method is practical and culturally acceptable as it represents the optimisation of existing local practices. The FB₁ level in the maize-based porridge, prepared from the maize

sorted and washed by the participants following training, was significantly ($p < 0.05$) reduced by 64% compared to the porridge prepared by the participants prior to training. The intervention study method effectively reduced the PDIs of FB₁ by 62% (21).

The tortillas the participants consumed in the Mexican study were prepared from maize kernels which were boiled in alkaline water followed by prolonged steeping in fresh water (19, 26). Subsequently, the cooked kernels were washed and ground to yield masa from which the tortillas were prepared. The cooking of kernels in alkaline water partially converts the fumonisins to their hydrolysed analogues and therefore the tortillas contain both fumonisins and hydrolysed fumonisins. The geometric mean UFB₁C (134 pg/mg creatinine) in the high tortilla consumption group in the Mexican study (19) was more than three-fold lower than at baseline in Centane (470 pg/mg creatinine). Based on the UFB₁ level in the high tortilla consumption group, FB₁ exposure was estimated to be 0.37 µg/kg body weight/day assuming a 1,500 mL/day urine output, a 60 kg body weight and an assumption of a 1% excretion of ingested FB₁ in urine based on data in swine and non-human primates (27, 28). It is notable that in the current study the measured urinary excretion of FB₁ was significantly lower than 1% (see below) and hence the intakes in Mexico may have been underestimated if urinary FB₁ excretion in the two populations is similar. Re-estimating the PDI in the Mexican study based on the estimated mean 24-h urine output and the urine excretion rate estimated for this study, the FB₁ PDI in Mexico (3.04 µg/kg body weight/day) was slightly lower than observed in Centane (4.83 µg/kg body weight/day). A recent study conducted in China with male and female participants reported median UFB₁C levels of 390 and 3,910 pg/mg creatinine,

respectively, in a high risk hepatocellular carcinoma area (Fusui) and in a high risk oesophageal cancer area (Huaian) (18). The median level reported for Fusui was similar to Centane (451 pg/mg creatinine), whereas the median level in Huaian was more than eight-fold higher.

The mean UFB₁ excretion in the Centane population based on individual body weight, an estimated urine output, the actual FB₁ content of the food consumed and the food consumption (dry weight) was 0.075%. The correlation between FB₁ intake and the urinary biomarker is influenced by the low proportion of FB₁ excreted in urine and the inter-individual variation of absorption and excretion of FB₁ (19, 28, 29). This variation was observed in vervet monkeys where toxicokinetic studies revealed individual variations in urinary excretion with values of 0.25, 0.66, 1.0 and 1.5% (28, 29). Experimental studies in rodents and swine reported urinary excretion of 0.4-2% (27, 30). A more recent study in swine reported a 0.9% urinary excretion with peak urinary excretion between 8 and 24 h following administration of a single oral dose of 5 mg FB₁/kg (31). In a preliminary human study in US participants consumed maize-based food that approximated maize consumption in urban Guatemala (32). The food was prepared from commercial masa and maize flour, containing 0.8 to 2.5 mg FB₁/kg. The urinary excretion of < 1% FB₁ measured in the US study was ten-fold higher than the < 0.075% measured in this current study. It remains possible that there are ethnic differences or food matrix effects which influence urinary FB₁ excretion in human populations. Future studies to measure FB₁ PDI in population would certainly be useful to improve our understanding on individual and ethnic variation in FB₁ excretion through urine.

Due to the rapid urinary excretion rate of FB₁, first void urine was collected 12 h following the last porridge meal consumed by the participants on two consecutive days at both baseline and intervention phases of the study. In a rat study following a single FB₁ gavage dose (25 mg/kg body weight) UFB₁ levels peaked at 12 h, whereafter levels declined rapidly, but FB₁ was still detectable 10 days later (30). This study also suggested that UFB₁ may reflect long-term exposure with chronic FB₁ exposure at low levels in rats (1.0 mg/kg body weight/day). Therefore, residual FB₁ from the previous days' maize consumption in the current study could have contributed to the measured UFB₁. Sources of FB₁ other than maize could also have contributed to the individual exposure. However, as this subsistence community is reliant on maize almost to the exclusion of all other food commodities, the contribution of other sources is expected to be insignificant. This was verified by utilising a validated questionnaire specifically designed for the local customary diet practices to obtain individual maize-based food consumption (33). The 52% reduction ($p < 0.05$) observed in UFB₁ (41% in UFB₁C) levels following intervention were comparable to the 62% reduction in FB₁ exposure as previously assessed by food analysis and food intake data (21). Indeed one of the advantages of assessing the intervention using a biomarker is that it provides a measure of the effect of the intervention on FB₁ exposure from all dietary sources whereas, by definition, the maize and food analysis indicates only the effect on the target commodity itself.

Conclusion

The FB₁ urinary biomarker is quantitatively correlated with FB₁ exposure at the individual level. The biomarker permitted confirmation of the reduction in fumonisin exposure achieved with the culturally acceptable intervention method of hand sorting

and washing maize. Utilisation of this biomarker will improve assessment of fumonisin exposure and thus contribute to the assessment of the possible health impacts of fumonisin exposure as well as permitting evaluation of intervention strategies to reduce fumonisin exposure. Future intervention studies could be expanded to include larger numbers of both male and female participants including children. Significant advances in food safety and health in subsistence maize farming communities exposed to high levels of fumonisin could be possible by further development of these approaches.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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7

Conclusion

The fumonisin levels in maize intended for human consumption from the State of Santa Catarina, Brazil, were similar to the high levels determined in other high oesophageal cancer incidence regions of the world where contaminated maize is consumed. The mean FB₁ level in maize samples from Santa Catarina intended for human consumption was 1.89 mg/kg, which is similar to levels reported in South Africa (2.10 mg/kg), Iran (2.27 mg/kg) and China (1.32 mg/kg) (Marasas 2001; Shephard et al. 2000; Yoshizawa et al., 1994). This is a further indicator for an association between the consumption of maize contaminated with fumonisins and oesophageal cancer.

The investigation into the disruption of sphingolipid biosynthesis in rats showed that FB₁-induced hepatocyte nodules were not resistant to the disruption of sphingolipid biosynthesis and is therefore not a major growth stimulus in their outgrowth. However, normal proliferating hepatocytes are more sensitive to the disruption of sphingolipid biosynthesis by FB₁ than quiescent hepatocytes. Although the inhibitory effect of FB₁ on ceramide synthase was reversible in hepatocyte nodules, sphingosine was apparently delayed in returning to initial levels. However, the delayed recovery effect of the sphingosine levels in the FB₁ -induced nodules compared to the surrounding tissue, and the sensitization of sphingosine accumulation in the nodules upon subsequent FB₁ exposure, could provide a selective growth stimulus possibly induced by bio-active sphingoid intermediates such as sphingosine 1-phosphate.

Previous studies conducted in the former Transkei region of the Eastern Cape Province of South Africa have shown that the oesophageal cancer incidence rate in

Centane is higher than in Bizana and that the home-grown maize consumed as the staple diet in Centane was contaminated with higher levels of fumonisins than in Bizana (Marasas, 2001; Rheeder et al., 1992; Shephard et al., 2007; Somdyala et al., 2003). Based on the mean total fumonisin levels in good home-grown maize, the fumonisin exposure in both Centane (2000) and Bizana (2000) exceeded the maximum tolerable daily intake for total fumonisins approximately three-fold as determined by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (Bolger et al., 2001). Since consumption of higher levels of fumonisin causes an increase in sphinganine and the sphinganine/sphingosine ratio in animal studies, it was hypothesised that the higher fumonisin exposure would result in higher sphinganine levels and sphinganine/sphingosine ratios in plasma and urine of participants in Centane than in Bizana. As postulated, the plasma sphinganine and sphingosine levels, as well as the sphinganine/sphingosine ratios of both the male and female participants, were higher in Centane than in Bizana. However, in contrast to previous studies conducted in these areas, the mean total fumonisin level in the maize collected contemporaneously with the blood and urine from these regions was lower ($p>0.05$) in Centane than in Bizana. The mean total fumonisin levels for Centane were similar to the lower levels reported for previous seasons in Centane, whereas the level for Bizana was higher than previously reported for Bizana. Further complicating the investigation into the long term effect of fumonisin exposure and the consequence on the sphingoid bases and their role as a biomarker in humans is the seasonal variation in fumonisin levels. Therefore subsequent studies were aimed at a comparison of sphingolipid biomarkers and fumonisin exposure on an individual basis.

The follow-up study was the first in Southern Africa assessing individual fumonisin exposure and concurrently evaluated sphinganine, sphingosine and sphinganine/sphingosine ratios in plasma and urine of each participant as possible biomarkers of fumonisin exposure. Individual fumonisin exposure was assessed by quantifying individual maize consumption with weighed food records and fumonisin levels from maize in each participant's household. The mean fumonisin levels of the first two seasons of the study were similar between the areas and only in the third season a five-fold higher mean fumonisin level was observed in Centane. The high consumption of 400 g maize/day (dry weight) in both areas confirms the reliance on maize as the dietary staple. The mean probable daily intake PDI in each area was approximately double the JECFA provisional maximum tolerable daily intake (PMTDI); with 20/59 participants exposed to levels exceeding the PMTDI, showing that certain individual participants were exposed to extremely high levels of fumonisin. Despite a very wide range of fumonisin exposure and direct comparison on an individual basis, sphinganine, sphingosine and sphinganine/sphingosine ratios failed to show a relationship with fumonisin exposure. Even comparison of the PDI of the participants exposed above and below the PMTDI did not show any difference in plasma or urinary sphingoid base profiles. Therefore, the sphingoid bases and ratios could not be associated with fumonisin exposure in this study and were negated as potential biomarkers of fumonisin exposure in humans.

The reduction of fumonisins in home-grown maize, based on customary methods of a subsistence farming community, was optimised under laboratory-controlled conditions. Hand-sorting by removal of only the most visibly infected or damaged kernels to reduce fumonisin contamination was possible due to the non-

homogeneous distribution of fumonisins among the kernels. A mean fumonisin reduction of 71% was achieved by selectively discarding only 2.5% of the infected/damaged kernels by weight of the original unsorted batch, which had a mean total fumonisin level of 2.32 mg/kg. Hence a small sacrifice in food quantity substantially improved the quality of the maize kernels for food preparation. The additional 13% reduction achieved with a 10 minute ambient water wash was deemed optimal, bearing in mind practicality and acceptability in the rural setting. Therefore, the optimised protocol consists of the removal of the infected/damaged kernels from the maize followed by a 10 minute ambient temperature wash with sufficient water to cover the maize. This recommendation was tested in an intervention study conducted in a subsistence farming community.

This intervention study was the first to report reduction in fumonisin exposure based on the customary maize-based food preparation practices in a subsistence farming community. The simple and culturally acceptable intervention method of sorting and washing as performed by the participants reduced fumonisin contamination in home-grown maize by 84%. The mean fumonisin level in the porridge prepared from the sorted and washed maize during intervention was reduced by 65% compared to the porridge prepared by the participants at baseline. Customised hand-sorting and washing of fumonisin contaminated-maize by the subsistence farmers to reduce fumonisin exposure was implemented effectively as fumonisin exposure, based on fumonisin levels and porridge consumption, was reduced by 62%. The simple two-step intervention method prior to the actual cooking of the maize did not prolong the food preparation time unduly and would thus be culturally sustainable. The intervention was implemented successfully, since fumonisin exposure was reduced

as assessed by food intake profiles and fumonisin food analysis in this subsistence farming community.

The urinary FB₁ biomarker study was the first to report a quantitative correlation between a fumonisin biomarker and individual exposure, thus validating the urinary FB₁ biomarker and confirming the reduction in fumonisin exposure at an individual level. The reduction achieved with the urinary FB₁ biomarker was 52% and when normalized with creatinine the urinary FB₁ it was 41%. Although the mean urinary FB₁ excretion was 0.08%, the reduced FB₁ exposures was significantly correlated with both the mean levels of both urinary FB₁ and the creatinine normalized urinary FB₁.

The biomarker was thus well correlated with fumonisin exposure and therefore confirmed the efficacy of the simple and culturally acceptable intervention method. Utilisation of the urinary FB₁ biomarker will improve assessment of fumonisin exposure and thus contribute to the assessment of the possible health impacts of fumonisin exposure as well as permitting evaluation of intervention strategies to reduce fumonisin exposure. Future intervention studies could be expanded to include larger numbers of both male and female participants including children. Significant advances in food safety and health in subsistence maize farming communities exposed to high levels of fumonisin could be possible by further development of these approaches.

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Addendum A

Total fumonisin levels in maize collected from the Centane and Bizana magisterial districts, Eastern Cape Province, South Africa can now be updated [Table 2 (p. 28) in the Literature Overview (Chapter 2)] following the publication of Van der Westhuizen 2008; 2010a; 2010b and 2010c (Tabel 1).

Addendum A - Table 1 Mean total fumonisin (FB₁ + FB₂ + FB₃) levels in maize intended for human consumption from Centane and Bizana collected over several harvest seasons.

Harvest Season	Centane		Bizana	
	n	Fumonisin (mg/kg)	n	Fumonisin (mg/kg)
1985 ^a	12	2.1 (nd–7.90)	12	0.083 (nd–0.55)
1989 ^a	6	1.63 (nd–6.70)	8	0.47 (nd–4.28)
1997 ^b	40	0.578 ± 1.28		
2000 ^b	41	0.882 ± 1.78	41	0.927 ± 1.71
2001 ^c	3	1.14 ± 0.575	7	1.02 ± 1.36
2002 ^c	9	0.404 ± 0.428	8	0.443 ± 0.61
2003 ^d	21	2.18 ± 2.27	36	0.356 ± 1.15
2007 ^e	6	2.32 ± 1.16		
2008 ^f	22	2.12 ± 1.37		

Values are means (range) or means ± standard deviation

nd = not detected, < 0.05 mg/kg

^a Rheeder et al., 1992 FB₁ + FB₂ only as the FB₃ standards was not available

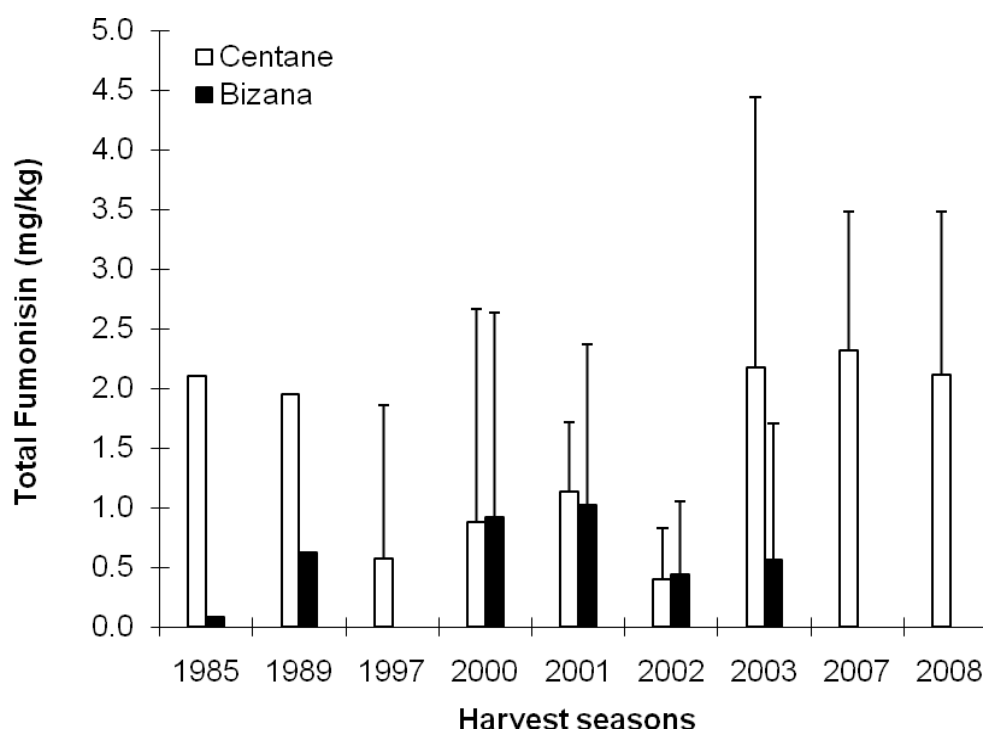
^b Van der Westhuizen et al., 2008

^c Van der Westhuizen et al., 2010a

^d Rheeder unpublished data

^e Van der Westhuizen et al., 2010b

^f Van der Westhuizen et al., 2010c



Addendum A - Figure 1 Mean total fumonisin levels in maize intended for human consumption from Centane and Bizana collected over several harvest seasons.

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Addendum B

Candidate's Contributions

Chapter 3.1 Fumonisin contamination and *Fusarium* incidence in corn from Santa Catarina, Brazil

- Primary author
- Generation of fumonisin data
- Analysis and interpretation of data

Chapter 4.1 Disruption of sphingolipid biosynthesis in hepatocyte nodules: selective proliferative stimulus induced by fumonisin B₁

- Primary author
- Conceptualisation and planning of study
- Wrote protocol for ethical approval
- Managed animal study and collected data
- Generation of sphingoid base data
- Analysis and interpretation of data

Chapter 5.1 Sphingoid base levels in humans consuming fumonisin contaminated maize from low and high oesophageal cancer incidence areas: a cross sectional study

- Primary author
- Planning of study
- Generation of fumonisin and sphingoid base data
- Analysis and interpretation of data

Chapter 5.2 Individual fumonisin exposure and sphingoid base levels in rural populations consuming maize in South Africa

- Primary author
- Planning of study
- Generation of fumonisin and sphingoid base data

- Analysis and interpretation of data

Chapter 6.1 Optimising sorting and washing of home-grown maize to reduce fumonisin contamination under laboratory-controlled conditions

- Primary author
- Conceptualisation and planning of study
- Wrote protocol for ethical approval
- Fieldwork: collection of data
- Generation of fumonisin data
- Analysis and interpretation of data

Chapter 6.2 Implementation of simple intervention methods to reduce fumonisin exposure in a subsistence maize farming community of South Africa

- Primary author
- Conceptualisation and planning of study
- Wrote protocol for ethical approval
- Fieldwork: collection of data
- Generation of fumonisin data
- Analysis and interpretation of data

Chapter 6.3 Fumonisin B1 as a urinary biomarker of exposure in a maize intervention study among South African subsistence farmers

- Primary author
- Conceptualisation and planning of study
- Wrote protocol for ethical approval
- Fieldwork: collection of data
- Generation of fumonisin data
- Analysis and interpretation of data

The following lists are additional contributions the candidate has made with regards to material covered in this manuscript.

Publication List

1. Burger HM, Lombard MJ, Shephard GS, Rheeder JP, Van der Westhuizen L and Gelderblom WCA. (2010) Dietary fumonisin exposure in a rural population of South Africa. *Food Chem Toxicol* 48: 2103–2108. (Impact factor 2.321)
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Presentations at Conferences

International

1. Van der Westhuizen L, Shephard GS, Burger HM, Rheeder JP, Gelderblom WCA, Wild CP and Gong YY. Reduction in Fumonisin Exposure by Practical Intervention Methods in a Rural Area of South Africa, 15th World Congress of Food Science & Technology, Cape Town International Convention Centre, Cape Town, South Africa, 22-26 August 2010, Oral
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